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translated from Russian

CONSULTANTS BUREAU

INTRAARTERIAL INFUSION OF PROCAINE

IN THERAPEUTIC PRACTICE

by N. K. Gorbadei

Translated from Russian

This English translation of Dr. Gorbadei's book includes as an appendix a report by I. I. Velikanov on treatment of hypertensive patients with intraarterial procaine infusions. Dr. Velikanov's report was first published in the Bulletin of the Academy of Sciences of the Kazak SSR.

Cases of peptic ulcer dating back to the siege of Leningrad responded to a series of infusions of procaine into the arteries, so that the patients were able to resume their normal activities, according to the author, Dr. N. K. Gorbadei who has reported on 177 cases of peptic ulcer treated at the Leningrad Sanitary-Hygiene Medical Institute.

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The Treatment of Peptic Ulcerations by Intraarterial Procaine Infusions [Clinical Features of the Patients Under Investigation; Influence on Subjective Symptoms; Roentgenological Findings During Treatment; Acid-secreting Function of the Stomach During Treatment; Changes in the Blood Picture; Changes in the Zones of Cutaneous Sensation; State of the Cardiovascular System (Electrocardiography and Plethysmography) During Treatment.]

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The Treatment of Patients with Hypertension by Intraarterial Infusion of Procaine Solution. I. I. Velikanov

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TRANSFORMATIONS OF UREA IN GASTRIC MUCOSA

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and Kh. Lind

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We concluded, from our earlier researches into the effect of administration of urea and glutamic acid to animals on the ammonia content of their gastric juice [1], that it probably arose from enzymic hydrolysis of urea within the gastric mucosal cells. This conclusion is in conflict with the views of a number of workers [2], who believe that this process takes place extracellularly, and is due to the urease of the gastric microflora; the ammonia so formed is thought to be absorbed by the mucosal cells, from which it enters the blood stream. The origin of gastric urease is thus still under question, and has been investigated by the authors of [3-7].

Fully convincing evidence of the bacterial origin of urease in animal organisms seemed to have been presented by the recently published work of Levinson et al. [8], who were unable to observe any breakdown of C^{14}-labelled urea administered to rats bred under sterile conditions. This finding is, however, in direct contradiction to that of Conway et al, [9], who showed that specimens of the contents of gastric mucosal cells, extracted from them with the aid of a microcannula, exhibited urease activity, although they were perfectly sterile, and gave no bacterial growth when inoculated into appropriate media. The contradictory nature of the findings of these two authors is, however, only an apparent one; it has been shown by a number of authors [10, 11] that urease is not present in the gastric mucosa of all animals. Thus it is present in humans, dogs, cats, and mice, but not in pigs, goats, frogs, some birds, and rats.

The work of Levinson et al. [8] thus only confirms earlier findings of the absence of urease from rat gastric mucosa, but can give no grounds for the generalization that in all mammals hydrolysis of urea is due exclusively to bacterial urease. It may be supposed that the presence or absence of urease in the gastric mucosa of different animals is related to the known peculiarities of secretion of gastric juice, in particular of HCl. Formation of HCl may depend on ion-exchange adsorption on structurally fixed intracellular macromolecular acids, such as proteins, including nucleoproteins and perhaps mucoproteins possessing acidic groups (carboxyl, phosphate, sulfate). Functioning as cationites, they would bind Na^+ and liberate H^+, which would, together with Cl^-, be secreted into the lumen of the pits as HCl. Ammonia, which is continually being formed as NH_4^+, and which shares many of the physicochemical properties of K^+ (low degree of hydration, etc.), is much more powerfully attracted by electrostatic forces to anions, and it immediately displaces Na^+ from the sphere of attraction of the electrostatic fields of the acidic groups of the intracellular structural cationites. The ammonia thus bound is, however, rapidly removed by incorporation into glutamic acid, to give glutamine; this process again exposes the acidic groups of the cationites, which can again bind Na^+, with liberation of H^+, one of the sources of which would be carbonic acid, formed by the action of gastric carbonic anhydrase on CO_2. Formation of HCl could be ascribed to this cyclic process of liberation and binding of ammonia. It is conceivable that glutamine would be the source of the ammonia in animals in which urease is absent from the gastric mucosa. A study of the comparative biochemical aspects of this problem would be of undoubted interest.

METHODS

The abdomen of dogs was opened aseptically under ether anesthesia, and the stomach was clamped at each end. Blood taken from the gastric vein was analyzed for ammonia and glutamine. Urea solution (5-10%) was then introduced from a large syringe, in amounts varying with the size of the stomach, so as to achieve an equal degree of filling in all cases. A sample (3-5 ml) was immediately withdrawn from the stomach, for determination of its ammonia content. Blood samples were again taken from the gastric vein for ammonia and glutamine determination, after 5 and 30 min, and samples of gastric contents were taken at the same times for ammonia determination. After completion of the experiment the clamps were removed, and the abdomen was closed. Ammonia was determined in the blood and gastric contents by Parnas' method, after precipitation of proteins by trichloroacetic acid, and amide nitrogen of glutamine was determined similarly, after preliminary hydrolysis with 2 N H_2SO_4. In many of the experiments blood urea was also determined by the xanthydrol method. The experiments were repeated on the same dogs after they had fully recovered from the operation, with the difference that instead of urea solution we introduced an equal volume of a solution of ammonium salts, of concentrations equal to, or greater than, the maximum values found in the urea solutions taken from the stomach in the previous experiments. Samples of blood and of gastric contents were taken in the same order, and at the same intervals, as before. In some of the experiments ammonium salts were introduced in the first experiment, and urea solution in the second.

EXPERIMENTAL RESULTS

The results presented in Table 1 show that there was a considerable rise (in some cases, a ten-fold rise) in the ammonia content of venous blood from the stomach following introduction of urea solution. The high initial levels of blood ammonia may be ascribed to the effects of the anesthetic [12]. The blood urea content rose parallel with the ammonia content, which is evidence of the rapid absorption of urea from the lumen of the stomach into the mucosal cells, and of its passage from there to the blood stream (Table 2). The ammonia content of the stomach contents also rose (Table 1). It could be supposed that the rise in the ammonia content of venous blood leaving the stomach was, in accordance with the views of the above-cited authors, due to absorption of ammonia formed in the lumen of the stomach by the action of bacterial urease on urea. We found, however, that (Table 1) introduction of ammonium salts into the stomach, at a concentration equal to that found during the experiments with urea, caused only an insignificant rise in venous blood ammonia levels; in some cases, no rise in blood ammonia could be observed. In one experiment, in which a 0.1 N NH_4Cl solution (140 mg-% NH_3-N) was introduced, this being ten times greater than the highest value found in urea solutions after introduction into the stomach, no rise in gastric venous blood ammonia was found. A considerable rise in blood ammonia followed rapidly after injection of urea solution into the stomach of the same dog in a subsequent experiment (Table 1, Expt. 8). The glutamine content of the venous blood similarly rose more, in most of the experiments, after introduction of urea solution into the stomach than after introduction of ammonium salts. It is of significance that the concentration of solutions of ammonium salts introduced into the stomach remained practically unchanged over the entire duration of the experiments (30 min); this points to the slow rate of absorption of ammonia from the stomach. No changes were found in the ammonia and the glutamine contents of the blood or the stomach contents following introduction of physiological saline (control experiment, Table 1, 3).

The pronounced rise in the ammonia and glutamine contents of venous blood from the stomach, following introduction of urea solutions, as compared with the very much smaller rise following introduction of ammonium salt solutions, leads to the conclusion that hydrolysis of urea takes place within the gastric mucosal cells, by the action of urease contained within them.

The problem of the origin of the ammonia found in the stomach contents following introduction of urea solution, and of the increase in its concentration, remains open. Little or no rise in the ammonia content of urea solutions introduced into the stomach was noted in many of the experiments 5 min after their injection, by which time a pronounced rise in blood ammonia was evident (Table 1, 3, 4, 7). It might have been supposed that the rise in the ammonia content of the urea solution in the stomach was due to the ureolytic action of bacteria present there. In order to check this possibility we washed out the stomach, through a temporary fistula, with three changes of bicarbonate solution, and then with 0.1 N HCl, after which we introduced 200 ml of 10% urea in 0.1 N HCl. Although Nikoloff [4] found that gastric mucosal urease differs from soybean urease in having a lower pH optimum, this is at pH 5, which excludes the possibility of enzymic decomposition of urea in a medium of pH 1. We however, obtained the following results: immediately after introduction into the stomach,

TABLE 1. Changes in the Ammonia and the Glutamine Contents of Gastric Venous Blood and of the Stomach Contents Following Introduction into the Stomach of Solutions of Urea or of Ammonium Salts. Expressed as mg-% of nitrogen

Serial No. of expt.	Solution introduced into the stomach	Blood						Stomach contents			
		NH_3 - N			amide nitrogen of glutamine			NH_3 - N			
		before introduction	5 min after	30 min after	before introduction	5 min after	30 min after	in solvent before introduction	immediately after introduction	5 min after	30 min after
1	Urea, 20 g in 200 ml water	0.16	0.29	0.72	1.38	1.53	1.86	—	1.84	3.38	6.70
	NH_4Cl, 200 ml (7 mg-% NH_3-N)	0.14	0.16	0.18	0.92	1.51	1.29	—	5.21	5.39	5.53
2	Urea, 20 g in 200 ml water	0.12	0.43	1.24	1.33	1.96	2.41	—	3.33	6.65	9.77
	NH_4Cl, 200 ml (14 mg-% NH_3-N)	0.16	0.17	0.17	1.16	1.83	1.57	13.68	10.16	11.40	13.16
3	Physiological saline, 150 ml	0.17	0.17	0.16	1.30	1.43	1.22	—	1.99	1.99	2.09
	Urea, 20 g in 200 ml water	0.18	0.48	0.63	1.72	2.05	3.11	—	3.40	2.72	7.52
	NH_4Cl, 200 ml (14 mg-% NH_3-N)	0.09	0.10	0.11	0.67	1.18	1.38	13.60	10.96	11.04	11.12
4	NH_4Cl, 200 ml (14 mg-% NH_3-N)	0.22	0.22	0.21	1.76	1.96	1.75	13.52	12.95	13.91	14.59
	Urea, 20 g in 200 ml water	0.19	0.54	1.96	1.77	3.58	4.61	—	2.77	3.67	11.63
5	NH_4Cl, 200 ml (14 mg-% NH_3-N)	0.22	0.23	0.19	1.34	1.40	1.85	14.04	14.70	14.54	14.46
	Urea, 20 g in 200 ml water	0.12	0.45	0.91	1.08	2.31	2.46	—	2.47	5.67	11.87
6	Urea, 3.75 g in 150 ml water	0.16	0.56	1.52	1.48	2.23	2.14	—	2.40	3.33	15.20
	NH_4HCO_3, 150 ml (15 mg-% NH_3-N)	0.08	0.08	0.10	1.25	1.32	1.34	18.52	18.40	18.08	18.80
7	NH_4HCO_3, 200 ml (150 mg-% NH_3-N)	0.08	0.10	0.18	1.87	2.19	2.32	16.56	15.60	16.00	15.60
	Urea, 10 g in 100 ml water	0.08	0.21	0.41	1.14	1.52	1.66	—	—	—	—
8	NH_4Cl, 200 ml (140 mg-% NH_3-N)	0.22	0.18	0.22	2.09	2.03	2.05	—	—	—	—
	Urea, 20 g in 200 ml water	0.34	2.09	3.98	1.92	5.61	5.83	—	2.34	5.52	16.00

TABLE 2. Changes in the Ammonia and Urea Contents of Venous Blood from the Stomach Following Introduction of Urea Solutions

Serial No. of expt.	Grams of urea introduced into the stomach	NH_3-N, mg-%			Urea, mg-%		
		before introduction	5 min after	30 min after	before introduction	5 min after	30 min after
1	20 (200)*	0.18	0.48	0.63	14.2	66.1	116.0
2	13.5 (135)	0.14	0.19	1.36	6.1	106.0	105.0
3	2.5 (100)	0.09	0.11	0.49	25.0	26.5	52.4
4	7.5 (150)	0.09	0.62	0.93	12.5	96.2	74.7
5	7.5 (150)	0.14	0.18	0.93	5.5	22.4	69.2
6	10 (200)	0.12	0.20	0.50	19.2	38.4	123.8
7	3.75 (150)	0.16	0.56	1.52	21.2	34.4	34.4

*Figures in parentheses refer to the volume of urea solution in ml.

TABLE 3. Section of Ammonia into the Lumen of the Stomach after Its Intravenous Injection

Serial No. of expt.	Introduced	NH_3-N, mg-%					
		stomach contents			blood		
		immediately after filling the stomach	5 min after intravenous injection of NH_4Cl	after 30 min	before intravenous injection of NH_4Cl	after 5 min	after 30 min
1	Into the stomach: 150 ml 0.9% NaCl Intravenously: 1.5 g NH_4Cl in 20 ml	2.08	2.12	2.84	0.15	4.00	1.22
2	Into the stomach: 300 ml distilled water Intravenously: 2.5 g NH_4Cl in 20 ml water	0.16	0.24	0.86	0.13	2.76	0.90

a urea solution contained 0.40 mg-% NH_3-N, after 5 min it contained 0.44 mg-%, and after 30 min 1.30 mg-%, i.e., increase in the ammonia content of the stomach contents was only observed after 30 min. The venous blood from the stomach, before introduction of acidified urea solution, contained 0.07 mg-% NH_3-N and 1.83 mg-% of glutamine amide-N; after 5 min the corresponding values were 0.14 and 2.37 mg-%, and after 30 min 0.22 and 3.14 mg-%.

Thus when a strongly acid urea solution was introduced into the stomach its ammonia content rose only slightly over the period of observation, whereas over the same time there was a pronounced rise in the ammonia and glutamic acid contents of venous blood from the stomach; this also constitutes evidence of its intracellular origin. Experiments were also performed, in which we incubated urea with extracts of gastric mucosa in 0.9% NaCl, cooling in liquid air during extraction. We found no increase in ammonia content, as compared with control systems not containing urea (2 ml of extract and 10 ml of 0.1 N HCl or 10 ml of 0.01 M urea in 0.1 N HCl). The results, expressed as mg-% NH_3-N were:

Time	Without addition of urea	With addition of urea
Before incubation	1.47	1.57
After 1 hr	1.57	1.62
After 2 hr	1.57	1.67

TABLE 4. Effect of Histamine on Formation of Ammonia from Urea in the Gastric Mucosa and on Its Secretion into the Stomach Contents. Nitrogen content, mg -%

Serial No. of Expt.	Injected intravenously	gastric mucosa		stomach contents		
		NH_3-N	amide nitrogen of glutamine	NH_3-N		
		30 min after the introduction of urea		immediately after introduction of water	10 min after	30 min after
1	5.4 mg histamine; 6.8 g urea	9.62	7.35	0.65	0.77	1.83
	8 g urea	3.98	4.80	0.55	0.69	1.08
2	4.8 mg histamine; 6.0 g urea	4.28	16.14	0.22	0.27	1.02
	4.5 g urea	2.14	4.66	0.26	0.24	0.28
3	1.0 mg histamine; 5 g urea	7.03	4.87	0.15	0.59	1.09
	5 g urea	4.88	5.12	—	0.19	0.58
4	1.0 mg histamine; 5 g urea	8.21	10.39	0.16	0.18	0.66
	5 g urea	2.57	6.73	0.15	0.25	0.53

The conclusion emerges from our findings that the ammonia appearing in urea solutions introduced into the stomach must have been secreted by the mucosal cells, in which it was formed, but not by the action of bacterial urease in the lumen of the stomach. Experiments were devised, with the object of testing this hypothesis, in which distilled water or physiological saline was introduced into the stomach simultaneously with NH_4Cl solution intravenously. Venous blood samples were taken immediately before these injections were made. The first sample of stomach contents was taken immediately after introduction of water or physiological saline, NH_4Cl solution was then injected into the blood stream, and samples of blood and of stomach contents were taken after 5 and 30 min (Table 3).

The data of Table 3 show that increase in the blood ammonia level is followed by a rise in the ammonia content of water or physiological saline present in the stomach. It would be reasonable to assume that ammonia would pass even more readily, and at a greater rate, into the stomach contents were it to be formed in the mucosal cells from urea and other sources. As for the biological significance of hydrolysis of urea in the gastric mucosa, it seems most probable that it is related to the secretory function of the stomach. The experiments of Table 4 were designed to provide additional support to our earlier evidence that a functional relation exists between formation of ammonia from urea and gastric secretion [1].

We introduced 60-80 ml of water, according to the size of the animal, into the stomach of a cat, clamping the stomach at both ends, and we immediately sampled the stomach contents. Histamine was then injected intravenously, followed after 5 min by an injection of urea solution, and samples of stomach contents were again taken after 10 and 30 min, for determination of ammonia content. The animals were then killed, and samples of gastric mucosa were rapidly frozen, and analyzed for ammonia and glutamine. For purposes of comparison, a similar experiment was performed with a second cat, with the sole difference that histamine was not administered, i.e., the secretory tissues of the gastric mucosa were in a resting state. Urea was given in doses related to unit weight of the animals.

The ammonia and the glutamine contents of the gastric mucosa were found to be higher in animals receiving histamine, and the ammonia content of the stomach contents rose accordingly, except in experiment 4 (Table 4), in which the ammonia content of the water present in the stomach did not exceed that found in the control animal, although it was higher in the mucosa, as in the other experiments. These results show that neurohumoral stimulation of secretion is, similarly to stimulation of the brain, associated with increased formation of ammonia.

SUMMARY

The ammonia content of gastric venous blood rises more after introduction of urea solution into the clamped stomach of a dog than when ammonium salts are introduced, under comparable experimental conditions. It is hence concluded that hydrolysis of urea, catalyzed by intracellular urease, preceeds in the gastric mucosal cells, with formation of urea.

Increase in the glutamine content of gastric venous blood indicates that the ammonia formed from urea re-enters the cycle of biochemical processes in the secretory cells of the stomach.

A comparison of the rise in the ammonia content of distilled water and of neutral or strongly acid urea solutions introduced into the stomach, following intravenous injection of ammonium salts, leads to the conclusion that the ammonia is released from the gastric mucosa, but does not originate from the action of bacterial urease in the lumen of the stomach.

Hydrolysis of urea provides a source of ammonia in the gastric mucosa; the reaction is of local significance only, and it enters into the biochemistry of gastric secretion, as is indicated by its intensification following histamine administration.

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DETERMINATION OF THE AMINO GROUP CONTENT OF CANAMYCIN, MYCERIN, AND COLIMYCIN

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In our earlier paper [1] we described the determination of the amino group content and molecular weight (mol. wt.) of the polypeptide antibiotics polymyxin M and V and gramicidin S, using the method of partial substitution, followed by electrophoretic separation of the reaction only to polypeptides possessing free amino groups. The same principles may be applied to the determination of the amino group content and mol. wt. of other classes of compounds. The present paper is devoted to a description of the determination of the amino group content of the antibiotics canamycin, mycerin, and colimycin, belonging to the neomycin group.

Of these antibiotics, canamycin has been the most extensively studied. Its molecule is made up of 6-glucosamine and canosamine (3-amino-3-desoxyaldohexose) residues, which are in glycosidic linkage with 2-desoxystreptamine. The molecule thus contains four primary amino groups. The mol. wt. of canamycin, as determined with the aid of a roentgenographic method, is 604 [2]. The antibiotics colimycin [3], discovered in the Institute for the Study of New Antibiotics, Academy of Medical Sciences, USSR, and mycerin [4], discovered in the Gamaleya Institute of Epideniology and Microbiology, belong to the neomycin group. Little is known about their chemical properties.

METHODS

Partial dinitrophenylation of canamycin on paper. To a solution of 10 mg of canamycin sulfate in 1 ml of water we added 50 mg of EDE-10-P anionite (OH form), the mixture was shaken for 20 min, and the anionite was separated by centrifugation. The solution of canamycin base was applied to a strip of chromatography paper 36 cm long (three spots, 2, 4, and 6 μl, in the middle of the strip, and 1 cm to each side of the center line). The spots were treated, in a stream of hot air, with an acetone solution of fluorodinitrobenzene (20 mg/ml), applying 0.6 mg per spot. The paper was then incubated for 25 min at 37°, and sprayed with an 85% HCOOH – CH_3COOH–water mixture (28:20:52). The wet strip was placed in position in an electrophoresis tank, and the current (220 v) was at once switched on; duration of electrophoresis – 2.5 hr. Dinitrophenyl derivatives of canamycin were stained yellow, while free canamycin was shown up by spraying with ninhydrin. The results of electrophoretic separation are presented in Table 1.

Partial dinitrophenylation of colimycin and mycerin was effected similarly to canamycin.

Preparation of dinitrophenylmycerin. To a solution of 50 mg of mycerin base in 2 ml of aqueous methanol and 0.1 ml of triethylamine we added 125 mg of fluorodinitrobenzene. The mixture was shaken for 2 hr at room temperature, and then incubated for 10 hr at 37°. Two ml of ether was then added to the orange-yellow solution. The precipitate was washed with 2 ml of ether, and was reprecipitated from acetone by adding ether. The yield of dinitrophenylmycerin amounted to 83 mg. It is readily soluble in acetone and nitromethane, sparingly soluble in glacial acetic acid and butyl acetate, and insoluble in ether, chloroform, cyclohexane, and alcohols. Deter-

mination of the optical density of weighed amounts of DNP-mycerin in acetone at 355 mμ served for the calculation of the equivalent of free mycerin, which was found to be 150-149. The molecular extinction coefficient of the dinitrophenyl group was taken as being 15,000.

RESULTS AND DISCUSSION

The applicability of the method of partial dinitrophenylation to determination of free amino group content of antibiotics of the neomycin group was tested for the case of canamycin, inasmuch as its structure has been established. The reaction was carried out on paper. This procedure had been worked out by us earlier for polymyxin M, and requires only very small amounts of antibiotic (about 100 μg is needed, in practice). Canamycin base was reacted with fluorodinitrobenzene in the absence of any neutralizing agent. Under such conditions the hydrofluoric acid evolved during the reaction combines with some of the amino groups of the antibiotic, and dinitrophenylation of these amino groups is thereby inhibited. The reaction hence ceases before all of the amino groups have been dinitrophenylated.

Electrophoretic separation of the mixture of reaction products showed the presence of four DNP-derivatives, of which one carried no charge, while the other three moved towards the cathode. In addition, the mixture contained free canamycin. The formation of four DNP-derivatives is evidence that the canamycin molecule has four free amino groups. The electrophoretic mobility of the partially substituted DNP-derivatives fell stepwise with increasing degree of substitution (Table 1). It is obvious that the spot which runs directly behind unsubstituted canamycin on the electrophoretogram must be mono-DNP-canamycin. Each of the electrophoretic fractions is a mixture of isomeric DNP-derivatives, differing in the position of the DNP-groups. Such isomers cannot, however, be separated electrophoretically. Tetra-DNP-canamycin is electrically neutral, and remains at the starting point during electrophoresis.

The values for the relative electrophoretic mobility of the DNP-derivatives are in good agreement with the theoretical values calculated for derivatives of a substance having four amino groups. Thus, the mobility fo mono-DNP-canamycin is three-quarters of that of free canamycin. This is as would be expected, since substitution of any of the four amino groups would reduce the charge carried by the antibiotic molecule in acid sclution by one unit. The number of free amino groups per molecule of canamycin found by our method of partial dinitrophenylation is in full agreement with the number shown by its structural formula.

Partial dinitrophenylation of mycerin and of colimycin, and electrophoretic separation of the resulting mixtures of products, were conducted under the same conditions as for canamycin. The mixture of partial dinitrophenylation products was found to contain six DNP-derivatives, pointing to the presence of six free amino groups in the mycerin molecule. The value found for the electrophoretic mobility of the DNP-derivatives is in good agreement with that calculated for a molecule with six free amino groups (Table 2).

The equivalent weight of DNP-mycerin was found to be 150, whence it follows that the molecular weight of mycerin is 900. Since, however, the molecular extinction coefficient used for the calculation of equivalent weight was that for DNP-amino acids, this may have introduced an error. Hence the value found by us for the mol. wt. of mycerin should be regarded as being an approximate one.

Partial dinitrophenylation of colimycin, followed by electrophoretic separation of the reaction products, gave the same results (Table 3) as for mycerin (Table 2). This antibiotic also has six free amino groups. Mycerin and colimycin behave very similarly on electrophoresis, and the same applies to their DNP-derivatives.

SUMMARY

It has been shown that the partial substitution method can be applied to the determination of the molecular weight of antibiotics of the neomycin class, which have aminosugars as components. The same method was used for determination of the free amino group content of canamycin, mycerin, and colimycin. We found that, in accordance with the literature, canamycin contains four amino groups. There are six amino groups in the molecules of mycerin and colimycin. The molecular weight of mycerin is about 900.

TABLE 1. Electrophoresis of the Products of Partial Dinitrophenylation of Canamycin

Derivative	distance of spot from starting point, cm (displacement towards the cathode)	relative mobility	
		found	calculated, for 4 amino groups
Canamycin	7.2	1.0	1.0
Mono-DNP-canamycin	5.4	0.75	0.75
Di-DNP-canamycin	3.6	0.50	0.50
Tri-DNP-canamycin	1.8	0.25	0.25
Tetra-DNP-canamycin	0.0	0	0

TABLE 2. Electrophoresis of Partial Dinitrophenylation Products of Mycerin

Derivative	distance of spot from starting point, cm (displacement towards the cathode)	relative mobility	
		found	calculated, for 6 amino groups
Mycerin	7.9	1.0	1.0
Mono-DNP-mycerin	6.9	0.87	0.83
Di-DNP-mycerin	5.7	0.72	0.67
Tri-DNP-mycerin	4.3	0.55	0.50
Tetra-DNP-mycerin	2.9	0.36	0.33
Penta-DNP-mycerin	1.5	0.19	0.17
Hexa-DNP-mycerin	0.0	0.0	0.0

TABLE 3. Electrophoresis of Partial Dinitrophenylation Products of Colimycin

Derivative	distance of spot from starting point, cm (displacement towards the cathode)	relative mobility	
		found	calculated, for 6 amino groups
Colimycin	7.8	1.0	1.0
Mono-DNP-colimycin	6.8	0.87	0.83
Di-DNP-colimycin	5.6	0.73	0.67
Tri-DNP-colimycin	4.3	0.55	0.50
Tetra-DNP-colimycin	2.8	0.34	0.33
Penta-DNP-colimycin	1.5	0.19	0.17
Hexa-DNP-colimycin	0.0	0.0	0.0

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N-TERMINAL AMINO ACIDS OF PEA LEGUMIN AND VICILIN

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It has been shown recently [1-4] that two globulins, legumin and vicilin, may be isolated from pea seeds by the method of isoelectric precipitation, and that these globulins are homogenous as shown by electrophoresis and sedimentation analysis. These proteins were also shown [1-4] to differ in their molecular weight, in their amino acid composition, and in a number of their physicochemical properties.

The present paper deals with the identification of their N-Terminal amino acids.

METHODS

The preparations of legumin and vicilin were isolated from pea seeds (Pisum sativum) of two varieties (Capital and Victoria), harvested in 1958, and grown in the Kishinev University experimental farm. The isolation and the electrophoretic testing for purity of these preparations have been described in an earlier paper [5]. The N-terminal amino acids were identified by the fluorodinitrobenzene method [6].

Dinitrophenylation was effected in the usual way: the protein was shaken at room temperature with a solution of fluorodinitrobenzene (FDNB) in 66% ethanol containing $NaHCO_3$. Full dinitrophenylation of the proteins was achieved only after 48 hr. Cater and Naismith [7] found that much the same applied to the dinitrophenylation of arachin.

We examined the possibility of applying Levy's method of dinitrophenylation [8], in aqueous solution at 40° and at pH 9, so as to accelerate the reaction. Carbonate buffer was used to maintain constancy of pH [9]. We supposed that under these conditions the protein would remain in solution, and that its reaction with FDNB would proceed faster. We found that DNP-vicilin did not separate out under the conditions of this procedure, while DNP-legumin precipitated out only partially after 2-3 hr of dinitrophenylation. However, the degree of dinitrophenylation of some of the N-terminal amino acids amounted to only 80% after 4 hr of reaction (taking 100% as the degree of dinitrophenylation achieved in alcoholic solution). There was, at the same time, an appreciable rise in the amounts of by-products formed, in particular, di-DNP-lysine from legumin, and di-DNP-lysine and di-DNP-tyrosine from vicilin. The yields of these products rose to 0.1-0.2 moles per mole of protein, whereas when dinitrophenylation was conducted in alcoholic solution only a few thousandths of a mole were produced. It may be concluded that dinitrophenylation of legumin and vicilin in aqueous solution under the specified conditions leads to partial breakdown of protein. In view of this finding the dinitrophenylation reactions on which our experimental data are based were conducted in alcoholic solution.

Hydrolysis of DNP-protein and treatment of the hydrolyzates. After dinitrophenylation the reaction mixture was acidified, and the precipitate of DNP-protein was collected on the centrifuge, and washed successively with water, alcohol, and ether. Washing with alcohol and ether was continued until a drop of the solvent no longer gave a dark spot when placed on filter paper and viewed under UV light.

A portion (about 10 mg) of DNP-protein was hydrolyzed for 12 hr at 105° in sealed, evacuated ampoules with 50 volumes of a 9:1 mixture of 6 N HCl and 85% formic acid. The formic acid was added in order to render the DNP-protein more soluble [8].

N-Terminal Amino Acids of Pea Vicilin and Legumin. The values given are the means of 10 determinations, and for DNP-glycine of 20 determinations

Protein	Mol. wt.	N-Terminal amino acids, moles per mole of protein*					mean mol. wt. per one N-terminal amino acid	number of polypeptide chains
		aspartic acid	glutamic acid	glycine	serine	leucine		
Vicilin	186 000	1.8	0.23	–	1.2	–	58 000	3
Legumin	410 000	0.30	0.20	7.8	–	4.1	33 000	12

*Applying a correction factor, amounting to 0.70 for DNP-aspartic acid, 0.70 for DNP-glutamic acid, 0.54 for DNP-glycine, 0.64 for DNP-serine, and 0.65 for DNP-leucine.

Hydrolysis of DPN-protein in 12 N HCl for 4 hr is usually recommended for the identification and estimation of DNP-glycine [8]. We found, however, in a number of experiments that neither free DNP-glycine nor DNP-glycine present in DNP-legumin underwent any more decomposition under our conditions of hydrolysis than when hydrolyzed for 4 hr in 12 N HCl. It is conceivable that the presence of formic acid protects DNP-glycine from decomposition (cf. [10]). The hydrolyzate was diluted with 4 volumes of water, and extracted with ether. The ether extracts were washed with N HCl, evaporated to dryness, and the residue was dissolved in acetone and transferred quantitatively to chromatography paper.

Chromatographic separation. We used Leningrad No. 4 chromatography paper. The basic solvent system used for separating ether-soluble DNP-amino acids was n-butanol – butyl acetate – 1% ammonia [11]. Matthias' radial-ascending procedure [12] gave better results than did ascending chromatography, used earlier [11, 13], and permitted of the separation of ether-soluble DNP-amino acids, dinitrophenol (DNP-OH), and dinitroaniline (DNP-NH_2), into the following ten groups (in order of rising R_f): DNP-aspartic and DNP-glutamic acids (do not move from starting point); DNP-serine; DNP-glycine; DNP-threonine; DNP-proline and DNP-alanine; DNP-OH; DNP-valine and DNP-methionine; DNP-phenylalanine, DNP-leucine, and DNP-tryptophan; di-DNP-lysine and di-DNP-tyrosine; DNP-NH_2. The solvent system used has the advantage over others that the DNP-OH spot is not superimposed on any of the DNP-amino acid spots.

For their further identification, the spots were eluted from the chromatograms [14], and were rechromatographed with phosphate buffer, pH 6 [15], or with a benzene – 1% acetic acid solvent [11]. The order in which the DNP-amino acids of groups 7, 8, and 9 ran in these solvents was about the same. A tendency towards trailing was observed with the second solvent system, but the degree of separation was usually adequate for the identification of the spots. An advantage of the second system was the relatively short time (4-5 hr) required for development. DNP-aspartic and DNP-glutamic acid were separated using the solvent system isoamyl alcohol – 1% CH_3COOH [14].

For the identification of DNP-leucine and DNP-isoleucine, which cannot be separated chromatographically, the spot corresponding with these DNP-amino acids was eluted, and the DNP-amino acids were hydrolyzed with concentrated NH_4OH [16]. The resulting amino acids were chromatographed, developing with butano saturated with citrate – phosphate buffer of pH 4.0 [17]. Treatment of the solutions of acid-soluble DNP-amino acids, and chromatography of the latter, were conducted according to the procedures dscribed in [13]. The spots on the chromatograms were located by viewing under ultraviolet light, using a PRK-4 quartz – mercury vapor lamp with a UFS-3 filter, and contact photographs of the chromatograms were taken.

Determination of DNP-amino acids after their elution from the chromatograms was effected by a polaroraphic method [18]. Correction factors for losses occurring during hydrolysis and chromatography were derived by subjecting known amounts of DNP-amino acids to the same treatment. The results were the same in the presence or the absence of DNP-proteins. The content of DNP groups in DNP-proteins was determined spectrophotometrically from the optical density at the absorption maximum (about 360 mμ) [19]. From these results, and taking into account the water content of the DNP-proteins, we determined the percentage protein content of the DNP-protein.

RESULTS

Fig. 1 shows chromatograms of the ether-soluble fraction of hydrolyzates of DNP-legumin and DNP-vicilin, developed in the system n-butanol – butyl acetate – 1% NH_4OH. Comparison with reference spots shows that the hydrolyzate of DNP-legumin contains DNP-glycine (spot 3), and that the hydrolyzate of DNP-vicilin contains DNP-serine (spot 2). These findings were confirmed by rechromatographing these spots with phosphate buffer as the solvent. Both hydrolyzates contained DNP-OH and DNP-NH_2 (spots 5 and 8). Eluates of spot 1 were developed with isoamyl alcohol – 1% CH_3COOH solvent (Fig. 2); the chromatograms showed the presence of DNP-aspartic and DNP-glutamic acids. Chromatograms of eluates of spot 6 of DNP-legumin hydrolyzate were run with the solvent system benzene – 1% CH_3COOH (Fig. 3) and with phosphate buffer showed that the spot may be due to DNP-leucine or DNP-isoleucine. Chromatograms of the amino acid regenerated from the DNP derivative established its identity as DNP-leucine (Fig. 4).

Spot 7 (initial chromatogram) is due to di-DNP-lysine in the case of legumin, and to di-DNP-tyrosine in the case of vicilin (Fig. 3). As has been mentioned, high yields of these DNP-amino acids are obtained only when proteins are dinitrophenylated in an aqueous system, evidently following breakdown of the protein during the reaction. Apart from the above, very faint spots, due to other DNP-amino acids, and not visible in the figures, were observed. Their content did not exceed 0.01 moles per mole of protein. A somewhat higher yield of DNP-serine from legumin, and of DNP-glycine from vicilin, was found; this may be ascribed to the presence of some vicilin in the legumin preparation, and of legumin in the vicilin preparation.

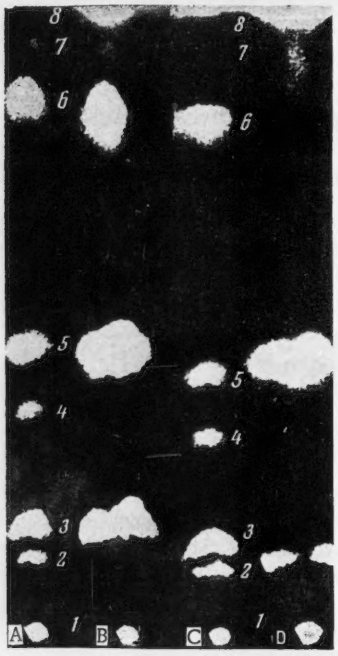

Fig. 1. Chromatograms of ether extracts of hydrolyzates of DNP-legumin and DNP-vicilin, using the solvent system n-butanol – butyl acetate – 1% NH_4OH (1:2:3) (initial chromatogram). A and C: reference mixtures; 1 – DNP-aspartic acid; 2 – DNP-serine; 3 – DNP-glycine; 4 – DNP-alanine; 5 – DNP-OH; 6 – DNP-leucine; 7 – see text; 8 – DNP-NH_2; B – DNP-legumin; D – DNP-vicilin.

Fig. 2. Chromatograms of eluates of spot 1 of the initial chromatogram developed with isoamyl alcohol – 1% acetic acid (1:1). A – DNP-vicilin, B – reference mixture: 1 – DNP-aspartic acid: 2 – DNP-glutamic acid; C – DNP-legumin. The spots at the top of the chromatogram are due to contamination with isoamyl alcohol, which absorbs in the UV.

Only E -DNP-lysine, O-DNP-tyrosine, and imidazole-N-DNP-histidine were found in the acid-soluble fraction. The following N-terminal amino acids have thus been identified: glycine, leucine, aspartic acid, and glutamic acid in legumin, and serine and glutamic acid in vicilin.

The results were qualitatively the same for both varieties of pea. The amount of legumin and vicilin prepared from the Capital variety of pea was insufficient for quantitative analysis. For this reason the values shown in the table relate only to the proteins isolated from peas of the Victoria variety.

The mean quadratic error of the mean values $\left(\sigma = \sqrt{\frac{\Sigma\Delta^2}{n(n-1)}}\right)$ for the determinations of DNP-amino acids and for the correction factors amounted to 2.5-3.0%.

The DNP-legumin was found to contain 83.2% of legumin, and the DNP-vicilin 82.7% of vicilin. These results are in good agreement with those calculated from the data for amino acid composition of legumin and vicilin [4], on the assumption that the reactive groups of lysine, tyrosine, and histidine are fully substituted. The mean error of determination of protein content of DNP-protein amounted to about 2%. Thus the overall error of the analyses did not exceed 8%. This does not, of course, exclude the possibility of a systematic error, resulting from differences in the rates of decomposition of free and DNP-protein-bound amino acids during hydrolysis.

We also determined the content of DNP-serine in DNP-legumin, and of DNP-glycine in DNP-vicilin, in order to assess the magnitudes of electrophoretically undetectable admixtures of vicilin in legumin, and of legumin in vicilin. We found (without dividing by the correction factor) 0.034 moles of DNP-serine and 0.021 moles of DNP-glycine per mole of DNP-proteins, which values correspond to contamination of legumin with 3.2%

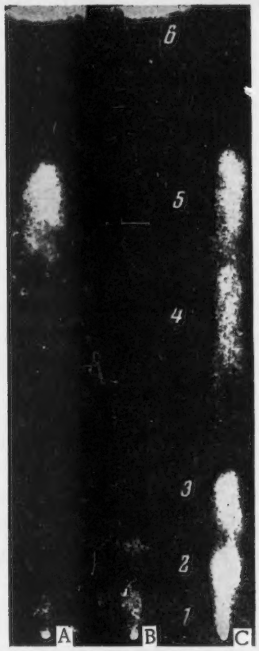

Fig. 3. Chromatograms of pooled eluates of spots 6, 7,and 8 of the initial chromatogram, developed in the solvent system benzene – 1% acetic acid (1:1). A – DNP-legumin; B – DNP-vicilin; C – reference mixture: 1 – di-DNP-lysine; 2 – di-DNP-tyrosine; 3 – DNP-tryptophan; 4 – DNP-phenylalanine; 5 – DNP-leucine; 6 – DNP-NH_2.

Fig. 4. Chromatograms of amino acids regenerated from spot 6 of the initial chromatogram of DNP-legumin, developed in butanol saturated with citrate – phosphate buffer of pH 4.0. Ascending chromatogram, run twice. A – amino acid under examination; B – the same + isoleucine; C – the same + leucine; D – isoleucine + leucine.

of vicilin, and of vicilin with 1.5% of legumin. These values are, however, probably higher than are the true ones, and should be regarded as expressing the upper limits of contamination of the proteins. As was mentioned above, other DNP-amino acids were yielded in very small amounts by both of the DNP-proteins; it is hence natural to suppose that some of the DNP-serine from legumin, and of the DNP-glycine from vicilin may have been due to the presence of such "natural" admixtures.

DISCUSSION

The N-terminal amino acids of vicilin were shown by our analytical results to be aspartic acid and serine, and of legumin to be glycine and leucine. Other N-terminal amino acids, such as aspartic acid and glutamic acid in legumin, and glutamic acid in vicilin, were in very small amount only. It is unlikely that they could have been due to breakdown of protein during dinitrophenylation, since their yields were the same under different reaction conditions. They most probably originated from some contaminating protein present in legumin and vicilin.

Our estimate, based on the results of N-terminal amino acid analysis, that legumin contained $<3.2\%$ of vicilin is in good agreement with the results of electrophoretic analysis of the legumin preparation [5]. As regards our sample of vicilin, electrophoresis showed that it contained a fraction, amounting to about 10% of the sample (fraction $\underline{x}$), which differed from the basic component in its mobility. The results of N-terminal amino acid analysis indicate that only a small proprotion of this fraction can be legumin. There is no reason to suppose that the N-terminal amino acids of this component differ from those of the basic protein component of vicilin (the small amount of N-terminal glutamic acid derived from vicilin can scarcely be ascribed to the presence of fraction $\underline{x}$, since it is also found in DNP-legumin hydrolyzates, whereas legumin does not contain fraction $\underline{x}$). We therefore believe that fraction $\underline{x}$ consists basically of vicilin, the electrophoretic mobility of which has been modified by some unrecognized factor.

The number of N-terminal amino acids per molecule of vicilin was calculated on the basis of Danielsson's value of 186,000 as the mol. wt. of vicilin [1]. In a later publication, however, this author [20] drew attention to the approximate nature of this value. This may explain why the deviations from whole integers of the number of N-terminal amino acid residues per molecule exceeded the experimental error. Rounding off the figures, we find that the vicilin molecule consists of three chains, of which two have N-terminal aspartic acid, and one N-terminal serine. The average mol. wt. of the chains is 58,000. The most recent publications [21-23] give the mol. wt. of legumin as being 410,000, as derived from sedimentation and diffusion constant measurements, or as 381,000 as derived from measurements of light scattering. The nearest approach to whole numbers for the contents of N-terminal amino acids is given if the mol. wt. is taken as 380,000. However, even if the mol. wt. is taken to be 410,000, the deviations from whole integers do not exceed the experimental error. It may be concluded from our determinations of the number of N-terminal amino acids in legumin that its molecule consists of 12 polypeptide chains, each of an average mol. wt. of 33,000.

It is of interest to compare our value for the mol. wt. of the chains with Brand and Johnson's findings [24] on the results of treatment of legumin with sodium dodecyl sulfate. These authors found that a product of mol. wt. 96,000 was formed, containing 0.5 molecules of dodecyl sulfate per molecule of protein. The mol. wt. of its protein moiety must therefore have been 64,000, which is twice the value for the mol. wt. of a polypeptide chain, as found by us. Dodecyl sulfate evidently degrades legumin to smaller aggregates, consisting of two polypeptide chains each.

Legumin differs markedly from vicilin in a number of respects: the nature of the N-terminal amino acids, the length of the constituent polypeptide chains, the average mol. wt. of which is nearly twice as great in vicilin as in legumin. On the other hand, legumin closely resembles arachin in a number of respects. Thus, although the number of N-terminal amino acids is greater in arachin [7], the basic ones are the same in both the proteins, viz., glycine and leucine, and the average mol. wt. of the polypeptide chains of arachin (32,000 [7]) is practically the same as of legumin (33,000).

SUMMARY

The N-terminal amino acids of pea legumin and vicilin have been identified and estimated by the fluorodinitrobenzene method. Vicilin was found to contain two N-terminal amino acids per protein molecule, two of which were aspartic acid, and one was serine; legumin contained eight N-terminal glycine, and four N-terminal leucine residues.

From our experimental data we derived the number of polypeptide chains constituting the protein molecules, and the average melecular weight of these chains. This value was found to be 58,000 for vicilin, and 33,000 for legumin. The degree of homogeneity of the protein preparations was evaluated from the results of N-terminal amino acid analysis.

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COMPARATIVE BIOCHEMICAL DATA FOR SOME FRESH-WATER INVERTEBRATES AND FISH

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The variety of forms of living organisms is associated with an equally great variety of biochemical peculiarities, both qualitative and quantitative. The individual types, classes, families, and even genera not infrequently possess some specific substances, characteristic of their biochemistry.

A considerable volume of data is now available, dealing with the comparative biochemistry of invertebrates and fish [1-10] (including fresh-water forms [10]).

The present paper is devoted to a more or less complete comparative biochemical analysis of certain fresh-water invertebrates and fish of a given biotope. We believe that the systematic prosecution of research of this kind in different aquatic biotopes could be of considerable value for the elucidation of the actual nutrients of fish, and of all the links in the nutritional chain of the organisms of the given biotope, as well as, possibly, of the conditions under which specific products are formed in the organisms.

MATERIAL USED

The animal material was collected during the summer of 1959 from the peripheral waters of the Rybinsk Reservoir. The water in these regions is of the calcium bicarbonate type, with a high content of dissolved organic matter and iron. The temperature of the water varies over the year from 0 to 22°. The bottom material is a gray, sandy clay.

The following species of fresh-water invertebrates were collected:

Insects: Dytiscus marginalis (imago) – a predator, not eaten by fish; Dytiscus marginalis (larvae) – predators, which feed on a variety of invertebrates, and are rarely eaten by fish; Tendipes plumosus (larvae) – feeds on bottom sludge and plant debris, and is the basic food of benthophage fish; Procladius (larvae) – predators, living mostly on Tendipes plumosus*; Notonecta glauca (imago) – predators, not eaten by fish; Phryganeae grandis (larvae) – predators, rarely eaten by fish; Enallagma cyatherum (larvae) – predators, sometimes eaten by fish.

Ticks: Hydrachnae comosa – a predator, never eaten by fish; Eylais sp. – a predator, never eaten by fish.

Worms: Limnodrillus newaensis – feeds on mud, and is a good nutrient for benthophage fish; Herpobdella nigricollis – a predator, eaten by fish.

Mollusks: Dreisena polymorpha – a typical microphage. Young individuals are readily eaten by benthophage fish.

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*According to the calculations of Luferov [11], up to 30 tons of Tendipes plumosus are consumed annually by Procladius per km^2 of the bottom of the Rybinsk Reservoir, whereas a typical benthophage, such as the bream, consumes only 0.3 tons. Procladius is also an acceptable nutrient of benthophage fish.

Fish: pike, ling, and bream, which differ widely from each other in their biochemical characteristics.

We collected 20-120 g dry weight of each species of invertebrate, and six specimens of each species of fish, taking the muscles, the liver, and the brain for analysis.

METHODS

Lipids were extracted in the following way: the sample (5-15 g) was vacuum-dried at 45° to constant weight, and the residue was ground up thoroughly with quartz sand. Lipoprotein complexes were decomposed by treatment with hot ethanol, and a weighed sample of the residue was extracted in a Soxhlet apparatus successively with ether, benzene, and chloroform (12 hr with each solvent), to extract lipids. Each fraction was analyzed for total fatty acids, total phospholipids, sphingomyelin, cholesterol, and lipid nitrogen. A portion of the fractions was hydrolyzed, for determination of galactose. Vitamin A was determined in a separate aliquot of the original dry residue, which had not been treated with hot alcohol.

Carbohydrates and proteins were extracted from 20-30 g of freshly homogenized material (after preliminary treatment with 80% alcohol, for inactivation of enzymes), successively with water, 10% NaCl, 80% alcohol, and 0.2% NaOH. The aqueous fraction was analyzed for albumins and globulins, histones, and carbohydrates, and the saline and alkaline extracts for nucleoproteins and histones. The alcoholic extract was evaporated down in vacuo to a syrupy consistency, and the residue was dissolved in water, and filtereJ. The insoluble residue represented alcohol-soluble proteins, while carbohydrates were in the filtrate. Proteins in the various fractions were precipitated in the following way: albumins and globulins by full - and half-saturation with $(NH_4)_2SO_4$, respectively, nucleoproteins by adding acetic acid in the presence of NaCl, or by adding alcohol and HCl, as recommended by Manoilov and Orlov [12], and histones by adding TCA. All the extractions were performed in the cold, with contiuous stirring by means of a magnetic stirrer for 6 hr. Portions of the fractions were hydrolyzed, for dtermination of galactose and mannose. Amino acids, fatty acids, and monosaccharides were identified and determined chromatographically (fatty acids were examined only qualitatively).

For these analyses a portion of absoultely dry material (1-2 g) was hydrolyzed with 20% HCl for 24 hr, and the HCl was distilled off in vacuo. Humins were removed by filtration. For the determination of DNA and RNA we applied the Schmidt – Thanhauser alkaline hydrolysis procedure [13].

For determination of water-soluble vitamins (B_1, B_2, and B_{12}) we extracted 20-50 g of freshly homogenized material with water for 12 hr, and then extracted the residue with three small portions of alcohol.

The following analytical methods were used.

Fatty acids were determined by Bloor's method [14], involving oxidation of lipids with oxidizing mixture, followed by measurement of optical density using a No. 7 filter. Phospholipids and sphingomyelin were determined by the method of Marenzi and Cardini [15]; the lipid phosphorus content (in mg), determined by the Fiske - Subbarow method, was multiplied by the factors 25.5 and 26.84, respectively. A No. 8 filter was used for the spectrophotometric measurements. Cholesterol was determined by the method of Feichmeier and Bergerman [16], involving precipitation as the digitonin complex, followed by color development with anthrone. A No. 3 filter was used in measuring the intensity of the color reaction.

Glycogen was determined by Kahan's method [17], with anthrone reagent; a No. 3 filter was used. Substances of the heparin group (mucoitinsulfuric acid and heparin) were determined by extraction of separate portions of material with 1% NaCl in weak ammoniacal solution, followed by precipitation with n-octylamine hydrochloride, extraction from the precipitate with phosphate buffer, and measurement of the coloration given with toluidine blue 0, using a No. 7 filter [18]. Chitin was determined in a portion of fresh material by Brach's method [19], from the nitrogen content of the chitin fraction, taking the nitrogen content of pure chitin to be 6.01%.

Proteins were determined by adding strong alkali to portions of the extracts, followed by 5% $CuSO_4$; the coloration due to the biuret complex formed was measured spectrophotometrically (No. 4 filter). When the extracts contained more than one protein (e.g., albumins and globulins), they were separated by fractionation with $(NH_4)_2SO_4$, as described above. Nucleoproteins were not analyzed as such – their content was derived from measurements of DNA and RNA phosphorus.

DNA and RNA were determined in the appropriate hydrolyzates by the method of Euler and Haph [20], by precipitation with lanthanum acetate, followed by determination of nuclein phosphorus by the Fisk – Subbarow

method [21]. The glycoprotein content was derived from the amounts of galactose and mannose liberated on hydrolysis. Nitrogen was determined by digestion with H_2SO_4, determining the ammonia formed colorimetrically, with Nessler's reagent [22] (No. 8 filter).

Vitamin A was determined by Vendt and Kuznetsova's spectrophotometric method [23], from its reaction with HCl and glycerol dichlorohydrin (No. 1 filter). Vitamin B_1 was determined by the simplified colorimetric method of Ostrovskii and Gvozdeva [24], with ninhydrin color reagent [No. 4 filter]. We tested the method on standard solutions containing protein; satisfactory results were obtained after precipitation of the protein with TCA, and adsorption of Vitamin B_1 on silica gel, followed by its elution; this procedure was followed in our analyses of animal material. Vitamin B_2 was determined by Trufanov's method [25], by elution from ascanite with pyridine mixture, followed by fluorescence measurement on a "Lumetron" fluorimeter. Vitamin B_{12} was determined by the chemical method, as modified by Bukin et al. [26], by extracting the dicyanide complex into benzyl alcohol, and measuring the optical density (filter No. 5).

Amino acids were analyzed chromatographically, as described by Ermakova [27], using No. 2B chromatography paper*, and staining the spots with ninhydrin. Monosaccharides (other than pentoses) were determined by Zaitseva and Afanas'eva's chromatographic method [28], using No. 4 paper (slow). Pentoses were determined in the filtrates after precipitation of protein with TCA, by the method of Roe and Rice [29], from the color reaction given by furfural with p-bromoaniline (No. 5 filter). Fatty acids were identified chromatographically according to Pustavalov [30], using No. 2B paper impregnated with a mixture of rubber and naphthalene in benzene.

For the photometric analyses we used a FÉKN-54 photoelectric colorimeter, and the results were read from standard curves constructed for each case.

RESULTS

The analytical scheme adopted by us allowed of a reasonably complete assessment of the biochemical difference existing between the relevant species of invertebrates and fish, and of their nutrient properties.**

Lipids

The mean values found by us for the contents of different classes of lipids are presented in Table 1. Of the invertebrates, Tendipes plumosus and Herpobdella nigricollis had the highest fatty acid contents, and Limnodrillus newaensis the lowest. The livers of fish had the highest fatty acid content, especially the liver of ling. The cholesterol content of invertebrates was low (except in the ticks). The cholesterol content of the liver and muscles of fish was also low, whereas the cholesterol content of the brain was quite considerable. Tendipes plumosus, Phryganeae grandis, and Enallagma cyatherum have very similar sphingomyelin contents; that of Dreisena polymorpha is much higher, whereas it was absent from the remaining invertebrates. Sphingomyelin is concentrated chiefly in the brain of fish, the highest content being in ling brain (0.840%). The contents of other phospholipids of invertebrates and fish vary over a wide range. Their content is high in Dytiscus marginalis (1.564%), Herpobdella nigricollis (3.512%), and in the brain of ling and bream (1.460 and 1.650%, respectively). A considerable fraction of the total phospholipids is due to sphingomyelin in Tendipes plumosus (34%), Enallagma cyatherum (28%), Procladius (22%), and Dreisena polymorpha (49%). In fish, the bulk of the phospholipids is made up of sphingomyelin (72% in ling muscles, for example). The phospholipids of invertebrates consist basically of lecithins, cephalins, phosphatidyl serines, and phosphatidyl inositols. No sphingomyelin was found in Dytiscus marginalis, Notonecta glauca, worms, or ticks. The cerebroside content of invertebrates was much lower than of the liver and brain of fish Limnodrillus newaensis is an exception to this rule, as it contains up to 0.030% of cerebroside galactose). Cerebrosides were absent from Notonecta glauca and from ticks.

*The designations given here and elsewhere in the text relate to chromatographic paper manufactured by the Volodarskii Factory, Leningrad.

**We could not, for reasons of economy of space, present all our data on the composition of the individual fractions as fully as was done for the amino acid composition of individual protein fractions; we therefore present only the mean values for the total contents of the particular substance.

The lipid nitrogen content of the liver and brain of fish is correspondingly high. No correlation can be observed between the lipid nitrogen content and those of total phosphalipids or cerebrosides. Nor does the lipid nitrogen content give any indication of the lipoprotein content.

The lipids of the animals studied also differ widely in the assortment of fatty acids contained in them (Table 2). Of them, we were able to identify only capric, stearic, hexadecenoic, oleic, and linoleic acids.

The fish tissues examined contained the same assortment of fatty acids as did the invertebrates (except for the muscles, which did not contain capric acid, or, in the pike, myristic and linoleic acids). Of all the acids identified by us, only two each could be found in Dytiscus marginalis, Notonecta glauca, and Phryganeae grandis. Linoleic acid was found only in Tendipes plumosus, Procladius, Limnodrillus newaensis, and Dreisena polymorpha; this acid is a vitamin for these organisms. Hexadecenoic acid was found only in Tendipes plumosus. The lipids of this group of invertebrates are thus distinguished by the narrow assortment of fatty acids entering into the composition of their lipids.

Carbohydrates

Our data on the contents of various carbohyrates in the species of animals examined by us are presented in Table 3. The glycogen content of invertebrates greatly exceeds that of fish, only the brain of which contains about as much glycogen as do invertebrate tissues. The highest glycogen content was found in Dreisena polymorpha (nearly half its dry weight). Notonecta glauca had the lowest glycogen content (4.644%).

The glycogen of fish is located chiefly in the brain and the liver. Substances of the heparin class were found in the liver and muscles of fish, while of the invertebrates only ticks and worms contained them, leeches having an exceptionally high content (0.424%). From 4 to 20% of chitin was found in the invertebrates, but it

TABLE 1. Comparative Data on Lipid Content (calculated as % of absolutely dry weight)

Species	Fatty acids	Cholesterol	Phospholipids			Cerebroside galactose	Lipid nitrogen
			sphingomyelin	others	total		
Insects							
Dytiscus marginalis (larvae)	12,830	0,000	0,000	1,564	1,564	0,004	0,001
" " (imago)	10,673	0,000	0,000	0,856	0,856	0,003	0,001
Tendipes plumosus (larvae)	19,673	0,091	0,219	0,419	0,638	0,002	0,012
Procladius (larvae)	7,500	0,074	0,117	0,422	0,539	0,012	0,011
Notonecta glauca (imago)	8,304	0,000	0,000	0,440	0,440	0,000	0,002
Phryganeae grandis (larvae)	4,350	0,184	0,238	1,592	1,830	0,010	0,011
Enallagma cyatherum (larvae)	3,060	0,037	0,349	0,631	0,980	0,010	0,013
Ticks							
Hydrochnae comosa	4,031	0,280	0,000	0,750	0,750	0,000	0,002
Eylais sp.	4,230	0,367	0,000	0,800	0,800	0,000	0,002
Worms							
Limnodrillus newasnis	1,580	0,000	0,000	0,100	0,100	0,030	0,014
Herpobdella nigricallis	12,300	0,181	0,000	3,512	3,512	0,018	0,009
Mollusks							
Dreisena polymorpha	9,800	0,059	0,455	0,475	0,930	0,011	0,004
Fish							
Pike muscle	6,530	0,160	0,054	0,131	0,185	0,003	0,011
Ling muscle	9,580	0,044	0,138	0,053	0,191	0,001	0,012
Bream muscle	8,760	0,031	0,150	0,082	0,232	0,009	0,001
Pike liver	7,540	0,038	0,082	0,109	0,191	0,013	0,045
Ling liver	25,831	0,006	0,080	0,241	0,321	0,011	0,018
Bream liver	13,840	0,004	0,075	0,108	0,183	0,013	0,014
Pike brain	10,580	0,584	0,654	0,907	1,561	0,038	0,045
Ling brain	11,345	0,930	0,840	1,460	2,300	0,061	0,154
Bream brain	9,541	0,450	0,750	1,650	2,400	0,072	0,031

was absent from Dreisena polymorpha. The monosaccharides produced by hydrolysis of nondefatted material included glucose, galactose, mannose, and pentoses, for both invertebrates and fish. The glucose content was closely correlated with the glycogen content. The galactose contents lay within only a narrow range, for both invertebrates and fish. The highest values were found in fish brain (up to 0.634% in the ling). The mannose content of invertebrates is much higher than of fish (Dreisena polymorpha is exceptional in not containing mannose); its content is closely correlated to that of chitin, of which it is not a component. The mannose of fish is present as a component of glycoproteins. Dytiscus marginalis larvae and bream muscles have a low pentose content. The highest pentose contents were found in fish brain (up to 0.20% in bream).

The aminosugar content of invertebrates differs markedly from that of fish. Whereas in the former the aminosugar nitrogen content may be as high as 0.650% (Dreisena polymorpha), the highest value found for fish was 0.07% (pike liver).

Proteins

An itemized analysis of the proteins of invertebrates is presented in Table 4. Fish proteins contain a higher proportion of globulins than do those of invertebrates. The lowest globulin content was found for Eylais sp. (13.577%), and the highest for the proteins of ling brain (60.800%). The albumin contents of fish and invertebrates were about the same; exceptions were for ling liver proteins (albumin content 24.154%), and for the proteins of the brain of fish (1.440-2.800% of albumins). It is of interest that, both in fish and in invertebrates, the lowest albumin content coincided with the highest globulin content, and vice versa. The albumin contents vary over a wider range than in the case of globulins; this is particularly evident in fish.

The content of basic proteins of the histone class is higher in invertebrates than in fish and is about the same for all invertebrate species examined (it varies widely in fish, from 0.0200 in bream liver to 1.850% in ling muscle).

The highest contents of nucleoproteins were found in the liver of fish (ling liver contained 2.614% of nucleoprotein phosphorus). High nucleoprotein contents were found in Procladius, Enallagma cyatherum and

TABLE 2. Comparative Qualitative Data on the Fatty Acid Assortment

Species	Capric acid	Myristic acid	Stearic acid	Hexadecenoic acid	Oleic acid	Linoleic acid
Insects						
Dytiscus marginalis (larvae)	+	−	+	−	−	−
" " (imago)	+	−	−	−	+	−
Tendipes plumosus (larvae)	+	+	−	+	+	+
Procladius (larvae)	+	+	−	−	+	+
Notonecta glauca (imago)	−	−	+	−	+	−
Phryganeae grandis (larvae)	−	+	−	−	+	−
Enallagma cyatherum (larvae)	+	+	−	−	+	−
Ticks						
Hydrachnae comosa	−	+	+	−	−	−
Eylais sp.	−	+	+	−	−	−
Worms						
Limnodrillus newaensis	−	+	+	−	+	−
Herpobdella nigricollis	−	+	+	−	+	−
Mollusks						
Dreisena polymorpha	+	−	+	−	+	+
Fish						
Pike muscle	−	−	+	+	+	−
Ling muscle	−	+	+	+	+	+
Bream muscle	−	−	+	+	+	+
Pike liver	+	+	+	+	+	+
Ling liver	+	+	+	+	+	+
Bream liver	+	+	+	+	+	+
Pike brain	+	+	+	+	+	+
Ling brain	+	+	+	+	+	+
Bream brain	+	+	+	+	+	+

TABLE 3. Comparative Data on Carbohydrate Content (expressed as % of absolutely dry weight)

Species	Polysaccharides			Monosaccharides				Amino-sugar nitrogen
	glycogen	heparin	chitin	glucose	galactose	mannose	pentose	
Insects								
Dytiscus marginalis (larvae)	8,964	0,000	8,364	7,384	0,409	0,044	0,014	0,430
Dytiscus marginalis (imago)	7,583	0,000	19,861	6,448	0,411	0,134	0,086	0,200
Tendipes plumosus (larvae)	14,861	0,000	6,584	11,354	0,422	0,036	0,034	0,124
Procladius (larvae)	17,310	0,000	4,383	13,864	0,400	0,018	0,058	0,104
Notonecta glauca (larvae)	4,644	0,000	11,800	3,585	0,290	0,064	0,136	0,280
Phryganeae grandis (larvae)	21,614	0,000	8,414	22,683	0,280	0,040	0,144	0,341
Enallagma cyatherum (larvae)	10,876	0,000	6,534	11,000	0,276	0,030	0,518	0,115
Ticks								
Hydrachnae com.	8,564	0,034	10,534	7,400	0,284	0,056	0,081	0,141
Eylais sp.	9,440	0,041	11,580	8,398	0,400	0,061	0,044	0,186
Worms								
Limnodrillus newaensis	6,584	0,058	9,841	5,990	0,436	0,048	0,038	0,344
Herpobdella nigricollis	10,615	0,424	8,533	9,344	0,451	0,040	0,045	0,440
Mollusks								
Dreisena polymorpha	44,380	0,000	0,000	40,134	0,401	0,000	0,090	0,650
Fish								
Pike muscle	0,856	0,134	0,000	0,330	0,433	0,020	0,091	0,018
Ling muscle	0,744	0,246	0,000	0,710	0,441	0,013	0,054	0,004
Bream muscle	1,534	0,181	0,000	1,503	0,453	0,022	0,011	0,010
Pike liver	2,583	0,934	0,000	2,534	0,441	0,032	0,028	0,070
Ling liver	3,160	0,243	0,000	3,034	0,421	0,023	0,034	0,012
Bream liver	4,616	0,139	0,000	4,030	0,429	0,021	0,035	0,012
Pike brain	5,860	0,000	0,000	6,536	0,628	0,019	0,181	0,004
Ling brain	3,410	0,000	0,000	7,344	0,634	0,013	0,156	0,010
Bream brain	9,845	0,014	0,000	10,844	0,627	0,012	0,200	0,003

Hydrachnae comosa. The proportion of DNA to RNA phosphorus varied for invertebrates from 1.9:1 for Hydrachnae comosa to 3.5:1 for Notonecta glauca, and for fish from 1.1:1 for pike liver to 3.5:1 for pike muscle.

The galactose and mannose contents of the protein fractions showed wide variations; in fish, the galactose and mannose contents show parallel variations, whereas no such regularity was found in the invertebrate group.

Table 5 gives the amino acid composition of the total protein content of invertebrates and fish. The data of this table show the absence of some amino acids from the proteins of both invertebrates and fish.

Thus, for example, cystine was not found in Dytiscus marg. (imago) or in Dreisena polymorpha, lysine was absent from Phryganeae grandis, aspartic acid from Limnodrillus newaensis, glycine from Notonecta glauca, serine from Eylais sp., alanine from Phryganeae grandis, Herpobdella nigricollis, and pike brain, glutamic acid from Eylais sp., tyrosine from Dreisena polymorpha, leucine from Herpobdella nigricollis, and phenylalanine from Dytiscus marginalis (imago and larvae), Hydrachnae comosa, and bream liver. The proteins of Dytiscus marginalis, Tendipes plumosus, Procladius, Notonecta glauca, and Dreisena polymorpha have high contents of lysine, glutamic acid, and leucine, those of Phryganeae grandis, Enallagma cyatherum, and Hydrachnae comosa are rich in glutamic acid and leucine, of Eylais sp. in leucine, of Limnodrillus newaensis in lysine, arginine, and leucine, and of Herpobdella nigricollis in lysine, histidine, and glutamic acid.

The proteins of fish muscle are rich in lysine, glutamic acid, and leucine, of fish liver in lysine, aspartic acid, and glutamic acid, and of fish brain in glutamic acid.

Thus the proteins of invertebrates and fish are distinguished by their high contents of lysine, glutamic acid, and leucine; the same applies to the proteins of mammals [31]. The high content of basic amino acids in the proteins of worms is correlated with their high content of histone. Cystine, arginine, glycine, serine, glutamic

TABLE 4. Comparative Data on Protein Content (expressed as % of absolutely dry weight)

Species	Globulin	Albumins	Histones	Phosphorus		Glycoprotein galactose	Glycoprotein mannose
				DNA	RNA		
Insects							
Dytiscus marginalis (larvae)	29,531	6,859	4,560	0,581	0,284	0,061	0,005
" " (imago)	33,839	4,839	3,830	0,432	0,133	0,028	0,026
Tendipes plumosus (larvae)	35,539	3,453	4,190	0,563	0,256	0,046	0,018
Procladius (larvae)	28,411	7,599	4,840	0,841	0,381	0,064	0,003
Notonecta glauca (imago)	25,000	10,769	2,490	0,382	0,110	0,125	0,001
Phryganeae grandis (larvae)	23,030	5,531	2,580	0,444	0,200	0,150	0,001
Enallagma cyatherum (larvae)	25,030	12,000	4,600	0,750	0,305	0,170	0,000
Ticks							
Hydrachnae comosa	15,020	12,500	4,720	0,833	0,430	0,090	0,002
Eylais sp.	13,577	12,880	2,809	0,483	0,200	0,026	0,024
Worms							
Limnodrillus newaensis	22,859	10,000	6,250	0,244	0,091	0,022	0,030
Herpobdella nigricollis	20,365	10,584	6,090	0,122	0,045	0,008	0,032
Mollusks							
Dreisena polymorpha	25,331	10,505	2,581	0,236	0,090	0,063	0,000
Fish							
Pike muscle	33,560	10,534	0,490	0,350	0,100	0,16	0,012
Ling muscle	49,850	10,800	1,850	0,450	0,154	0,238	0,013
Bream muscle	55,660	5,000	0,440	0,134	0,086	0,364	0,016
Pike liver	33,854	9,330	0,651	1,000	0,944	0,334	0,014
Ling liver	24,859	24,154	0,830	1,856	0,758	0,230	0,013
Bream liver	45,601	6,660	0,020	1,040	0,486	0,034	0,001
Pike brain	54,750	2,800	0,100	0,950	0,330	0,341	0,015
Ling brain	60,800	1,440	0,050	0,360	0,190	0,134	0,006
Bream brain	34,900	4,000	0,205	0,800	0,238	0,300	0,012

acid, valine, methionine, and phenylalanine are more or less uniformly distributed in the proteins of the orgains of fish. The contents of other amino acids in the proteins of the different organs vary, according to the source of the protein. The highest contents of lysine, histidine, and aspartic acid are encountered in the liver proteins, of tyrosine in the cerebral proteins, and of leucine in the muscle proteins. The muscle proteins of the different fish have a more or less equal alanine content, which varies much more from species to species for brain and liver protein.

VITAMINS

The mean results for the vitamin contents of invertebrates and fish are presented in Table 6.

Of the invertebrates only Tendipes plumosus and Procladius contained all the vitamins under survey, mostly A_1, B_1, and B_{12}. The highest content of B_2 was found in Dreisena polymorpha (0.835 mg-%). Vitamin A was absent from Dytiscus marginalis and from worms, vitamin B_1 from Dytiscus marginalis (imago), vitamin B_2 from Dytiscus marginalis (larvae) and Herpobdella nigricollis, and vitamin B_{12} from Dytiscus marginals (imago), Dreisena polymorpha, and ticks. Of the species of fish examined, vitamin A was absent from ling muscle and brain, vitamin B_1 from the muscles of all three species, vitamin B_2 from the muscles and liver of ling, and vitamin B_{12} from ling muscle and bream liver and brain. The highest vitamin A content was found in ling liver (1.340 mg-%), of vitamin B_1 in bream brain (0.516 mg-%), of vitamin B_2 in pike brain (0.314 mg-%) and of vitamin B_{12} in pike liver (0.214 mg-%).

TABLE 5. Comparative Data for the Amino Acid Composition of Proteins (expressed as % of Protein)

Species	Cystine	Lysine	Histidine	Arginine	Aspartic acid	Glycine	Serine	Glutamic acid	Alanine	Tyrosine	Valine	Methionine	Leucine	Phenylalanine
Insects														
Dytiscus marginalis (larvae)	2,000	11,844	3,866	6,600	6,391	3,366	5,100	19,368	6,376	3,990	3,010	2,841	9,733	0,000
" " (imago)	0,000	12,000	3,866	9,090	7,544	1,980	5,500	10,000	10,000	4,086	3,120	3,000	15,800	0,000
Tendipes plumosus (larvae)	3,864	13,836	2,986	8,364	9,789	2,950	5,300	20,384	7,584	0,440	3,650	4,310	10,483	5,300
Procladius (larvae)	2,434	11,344	3,110	8,584	8,654	3,019	6,090	19,754	6,936	3,956	3,531	3,651	14,651	2,410
Notonecta glauca (imago)	4,486	4,364	4,000	2,687	7,876	0,000	2,000	18,383	6,674	5,000	3,000	4,900	12,200	4,850
Phryganeae grandis (larvae)	5,056	0,000	6,340	2,311	6,400	6,038	5,100	20,383	0,000	5,300	2,856	1,815	11,000	5,455
Enallagma cyatherum (larvae)	5,134	2,586	5,860	8,300	7,000	3,010	4,900	26,376	4,000	6,100	2,734	1,754	10,539	4,750
Ticks														
Hydrachnae comosa	4,225	1,002	6,410	8,418	3,542	2,865	5,100	22,383	1,186	5,397	2,000	1,650	12,825	0,000
Eylais sp.	4,300	8,100	6,400	8,400	12,000	2,765	0,000	0,000	8,300	4,958	2,850	1,700	13,100	4,844
Worms														
Limnodrillus newaensis	2,086	11,056	3,330	12,841	0,000	2,834	5,800	9,397	8,561	4,184	2,440	2,563	13,343	4,234
Herpobdella nigricollis	3,033	10,000	15,833	10,564	4,861	8,938	5,750	13,241	0,000	5,041	2,021	3,381	0,000	4,000
Mollusks														
Dreisena polymorpha	0,000	13,586	4,654	6,734	3,000	2,333	5,330	12,839	6,440	0,000	0,000	4,053	18,564	3,500
Fish														
Pike muscle	1,456	10,854	1,258	7,877	8,900	2,560	3,910	22,333	6,811	3,411	3,844	3,563	15,400	4,300
Ling muscle	1,839	11,354	1,353	6,933	7,000	2,060	3,744	21,831	5,990	3,518	2,312	4,010	10,400	5,611
Bream muscle	1,731	9,763	1,853	5,854	7,659	2,133	3,133	20,534	6,010	2,440	6,114	3,000	11,676	4,054
Pike liver	1,683	18,110	3,488	6,564	13,840	3,440	5,480	14,886	0,956	3,860	5,061	3,311	5,460	5,610
Ling liver	1,534	17,665	3,584	6,400	13,414	2,800	0,330	10,534	9,544	8,130	4,038	3,280	6,280	4,000
Bream liver	1,200	15,451	2,733	6,784	12,560	2,000	1,800	20,814	3,841	4,000	0,980	3,056	8,000	0,000
Pike brain	1,884	7,641	1,534	7,663	5,811	1,450	2,711	28,767	0,000	6,540	3,031	3,561	2,000	5,800
Ling brain	1,936	6,384	1,053	6,581	4,391	2,344	3,000	27,064	0,987	5,431	2,810	4,663	3,455	4,000
Bream brain	1,800	7,000	1,943	6,443	6,954	3,856	4,564	25,039	2,544	5,861	3,000	3,841	3,338	0,900

DISCUSSION OF RESULTS

Notwithstanding the principle of the unity of biochemical organization of animals, each zoological group of organisms has its own biological peculiarities. Widely differing groups of animals, such as fish and insects, or mammals and sponges, frequently possess their own specific types of proteins, fats, carbohydrates, and intermediary metabolites, encountered only in the given group (chitin in arthropods, spongine in sponges, cellulose in tunicates, etc.). More closely related groups (species, genera, families) are less frequently distinguished from each other by the possession of some specific component – the differences are more usually of a quantitative nature only. Finally, organisms of a given species may contain proteins, fats, and carbohydrates of the types specific for the given species, but in different proportions, depending on the available nutrients and on the environmental conditions of their biotope. Differences in the mineral composition of animals, in particular as regards trace elements, are often much greater than are the differences in their contents of organic components, and this applies to both the qualitative and the quantitative aspects.

Any change in the chemical composition of the environment must also affect the chemical composition of the organism living in this environment, leading to changes in its metabolic processes, often to development of morphological modifications, and, under extreme conditions, even to its death [32, 33]. A whole series of examples of morphological variabilty in plants and animals [33] have been revealed by recent studies on biogeochemical provinces. We are thus in agreement with the view advanced by Florkin [1], that variation in the forms of animal organisms are a consequence of their biochemical differences, in particular of their metabolism.

Our findings provide evidence of the existence of considerable differences in the contents of fats, carbohydrates, proteins, and vitamins among the various species of invertebrates and fish examined. Qualitative differences were also revealed. Since the environmental conditions and the chemical composition of the biotope were constant, and common to all the organisms, our data relating to the chemical composition of the animals may be regarded as expressions of their biochemical peculiarities, under the conditions of the given biotope.

Accumulation of fats in the bodies of invertebrates does not appear to bear any relation to their mode of life or of nutrition. Tendipes plumosus, which feeds on mud and vegetable debris, and Dytiscus marginalis and Herpobdella nigricollis, which are predators, have practically identical fatty acid contents. These acids, however, exhibit considerable qualitative differences. Linoleic acid, for example, was not found in the predatory invertebrates, but was present in the detritophagous, phytophagous, and microphagous forms. It should be noted that our findings for the total fat contents of the organisms examined are in agreement with published data [34]. Some interest attaches to the presence of cholesterol in ticks, but not in any other group of invertebrates examined. All the invertebrates had about the same phospholipid content (with the exception of Limnodrillus newaensis, which contained 0.1%), but the proportions of the component phospholipids showed very wide variations, as is evident from the data for sphingomyelin. This may be absent altogether (Dytiscus marginalis, Notonecta glauca, ticks, worms), or it may constitute a large proportion (up to 34%) of the total phospholipids (Tendipes plumosus). No correlations could be discerned between the fatty acid, phospholipid, cerebroside, and lipid nitrogen contents of the animals.

Fish are in general richer in lipids than are invertebrates; the liver contains more fats, and the brain more lipoids. The phospholipids consist largely of sphingomyelin, and there is a more varied assortment of fatty acids.

The glycogen content of invertebrates varies over a wide range [35]. Of particular interest is the presence of heparin-like substances in ticks and worms, amounting to as much as 0.424% in Herpobdella nigricollis. They were not present in mollusks and insects. Chitin was found in all the invertebrates, with the exception of Dreisena polymorpha, and its content was highest in the adult forms. As for fats, glycogen is mobilized during metamorphosis, showing that it serves as a reserve substance [36]. Only an insignificant part of the galactose present in invertebrates enters into the composition of cerebrosides. Glycoprotein galactose varies over a wide range. Part of the galactose appears to be present as a component of some as yet unidentified substances (amines).

The mannose content appears to be correlated with the chitin content, of which it is a component (Dreisena polymorpha, which does not contain chitin, also contains no mannose). Part of the mannose is present in glycoproteins, and was found by us in the protein fractions.

It is evident from a comparison of the proteins of fish and invertebrates that fish have a much higher globulin and nucleoprotein content, but a lower histone content, than do invertebrates. The albumin contents

TABLE 6. Comparative Data on Vitamin Content (expressed as mg-% of absolutely dry tissue)

Species	Vitamin			
	A	B_1	B_2	B_{12}
Insects				
Dytiscus marginalis (larvae)	0,000	0,983	0,000	0,054
" " (imago)	0,000	0,000	0,058	0,000
Tendipes plumosus (larvae)	1,383	1,814	0,131	0,081
Procladius (larvae)	0,764	0,844	0,244	0,034
Notonecta glauca (imago)	0,085	0,031	0,091	0,000
Phryganeae grandis (larvae)	0,090	1,561	0,000	0,033
Enallagma cyatherum (larvae)	0,090	1,360	0,100	0,041
Ticks				
Hydrachnae comosa	0,085	0,081	0,100	0,000
Eylais sp.	0,110	0,090	0,100	0,000
Worms				
Limnodrillus newaensis	0,000	0,154	0,831	0,014
Herpobdella nigricollis	0,000	0,314	0,000	0,080
Mollusks				
Dreisena polymorpha	0,254	0,483	0,838	0,000
Fish				
Pike muscle	0,054	0,000	0,098	0,056
Ling muscle	0,000	0,000	0,044	0,000
Bream muscle	0,048	0,000	0,000	0,031
Pike liver	0,954	0,344	0,010	0,214
Ling liver	1,340	0,258	0,244	0,181
Bream liver	0,334	0,154	0,000	0,000
Pike brain	0,054	0,400	0,314	0,056
Ling brain	0,060	0,400	0,200	0,044
Bream brain	0,003	0,516	0,256	0,000

are very similar in both groups of animals (the brain, however, has a much lower albumin content than do the other tissues examined).

A certain correlation may be perceived between the histone and the nucleic acid phosphorus contents of invertebrates are of the nucleohistone type, whereas those of fish also contain albumins. This view is supported by the deficiency in albumin in brain proteins, against a background of high nucleic acid phosphorus. The DNA:RNA ratios were fairly constant, and ranged from 3.5:1 to 1.1:1.

Considerable differences were found in the vitamin contents of invertebrates. They were plentiful in Tendipes plumosus and Procladius, and this, in conjuction with their high and varied contents of lipids, carbohydrates, and proteins, and with the high biological value of the amino acids present in the latter, renders these organisms excellent foods for benthophage fishes. In contrast, predatory invertebrates are poorer in vitamins (Dytiscus marginalis contains only one vitamin – B_2, and this in only small amount). Our findings coincide in part with those of Malikova and Fisher [34, 37]. In fish the vitamins are located mostly in the liver (ling liver is exceptional in not containing vitamin B_{12}). Fish muscle is poor in vitamins (thiamin was not found at all).

SUMMARY

The biochemical singularities of certain fresh-water organisms, living under the conditions of a single biotope, have been found, for different zoological classes, to be represented not only by quantitative differences in biochemical composition, but also by the presence or absence of particular compounds.

The invertebrates had the most complex assortment of lipids, whereas the fish had the most varied assortment of fatty acids. The lipid content of fish was in general higher than of invertebrates.

The carbohydrates of fish differed from those of invertebrates in having a higher proportion of heparin and heparin-like substances, and in the absence of chitin. In contrast to the organs of invertebrates, those of fish (except for the brain) had a low glycogen content. Invertebrates had a higher aminosugar content than did fish; this difference is related to the presence of chitin in the former class of organisms.

The correlation found between the histone and the nucleic acid phosphorus contents of invertebrates supports the view that in these organisms nucleoproteins are represented chiefly by nucleohistones. The nucleoproteins of fish appear to consist chiefly of compounds of nucleic acid with other proteins, possibly albumins. The DNA:RNA ratio was practically constant for all the invertebrates examined.

It was concluded, from the data for their biochemical composition, that detritophage, phytophagous, and microphagous organisms should have the highest nutritional value for benthophage fish; these included Tendipes plumosus, Procladius, Limnodrillus newaensis, and Dreisena polymorpha.

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INHIBITION OF ENZYMIC DECARBOXYLATION OF AMINO ACIDS BY DL-PENICILLAMINE, L-CYSTEINE, AND DL-HOMOCYSTEINE

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Inhibition of aminopherase activity of animal tissues by L-penicillamine has been demostrated by du Vigneaud et al. [1, 2], both in vivo and in vitro, and the explanation of the inhibition mechanism as being due to formation of a thiazolidine ring structure with pyridoxal phosphate and penicillamine [2] is of considerable theoretical and practical importance.

The elucidation of the mechanism of inhibition by penicillamine gives grounds for the generalization that penicillamine should be an inhibitor of all enzymic systems involving pyridoxal phosphate as coenzyme. It has been shown that the serine dehydrase activity of Neurospora crassa is also powerfully inhibited by penicillamine [3]. Our preliminary experiments have shown, as was to be expected, that penicillamine inhibits decarboxylation of amino acids by microbial enzyme systems [4]. Further investigations have shown that not only penicillamine, but also cysteine and homocysteine are powerful inhibitors of enzymic decarboxylation of amino acids.

EXPERIMENTAL

Enzyme preparations were made from microorganisms which decarboxylate amino acids, viz., Streptococcus faecalis (tyrosine decarboxylase), Micrococcus sp. n. (histidine decarboxylase), E. coli (arginine decarboxylase), B. cadaveris (lysine decarboxylase), Mycobacterium sp. n. (aspartic decarboxylase), and Cl. welchii S. R. -12 (glutamic decarboxylase). For this purpose we made dry acetone powder preparations of the bacteria, by the procedure previously described [5].

The experiments were performed in a Warburg apparatus at 30°. We placed a suspension of 15 mg of acetone powder in 2.5 ml of buffer solution into the main compartment of the Warburg vessel, with inhibitor (DL-penicillamine,* L-cysteine, or DL-homocysteine), and equilibrated for 15 min, after which a solution of 8.3 μmoles of amino acid in 0.5 ml of buffer (M/60) was tipped in from the side-tube; the rate of decarboxylation was then measured.

The results of our experiments, presented in Tables 1-7, show clearly that penicillamine, cysteine, and homocysteine strongly inhibit enzymic decarboxylation of amino acids. Inhibition by penicillamine (Tables 1-3) is ascribable to formation of biologically inactive thiazolidine compound with the coenzyme pyridoxal phosphate, as has been conclusively demostrated by du Vigneaud et al. [2].

It may be supposed that inhibition by cysteine (Tables 4-6) is due to some analogous reaction mechanism. In any case, it is know that the purely chemical reaction of condensation of pyridoxal with cysteine proceeds as

*We take this opportunity of thanking Dr. Chem. Sci. O. V. Kil'disheva for supplying us with a preparation of DL-penicillamine.

TABLE 1. Effect of Addition of DL-Penicillamine (in μ moles per system) on Decarboxylation of L-Aspartic acid by Mycobacterium sp. n. and of L-Histidine by Micrococcus sp. n. Acetate buffer, pH 5.6. In this and the following tables: total volume of system 3 ml, containing 15 mg of microbial acetone powder and 8.3 μmoles of amino acid. Results as μl of CO_2.

Reaction time, min	Mycobacterium sp. n. + aspartic acid				Micrococcus sp. n. + histidine			
	0,25	2,5	25	control	0,25	2,5	25	control
10	176	172	151	178	80	74	49	80
20	183	177	172	185	118	107	82	115
30	189	183	177	188	148	138	108	142
40	190	183	178	188	164	155	127	169
50	190	183	178	188	176	166	140	183
60	—	—	—	—	184	168	143	186

TABLE 2. Effect of Addition of DL-Penicillamine on Decarboxylation of Lysine by B. cadaveris. Penicillamine content of systems in μmoles

Reaction time, min	Phthalate buffer, pH 5.50				Phosphate buffer, pH 6.0			
	0.25	2.5	25	control	0.25	2.5	25	control
10	81	67	37	85	80	68	34	73
20	140	127	81	150	124	110	63	120
30	157	150	104	170	138	128	80	136
40	158	156	119	173	143	136	100	142
50	160	159	127	175	143	136	100	142
60	160	159	130	175	143	136	101	142

TABLE 3. Effect of Addition of DL-penicillamine on Decarboxylation of Arginine by E. coli. Penicillamine content of systems in μmoles

Reaction time, min	Citrate–phosphate buffer, pH 5.2				Acetate buffer, pH 5.6			
	0.25	2.5	25	control	0.25	2.5	25	control
10	38	25	0	45	77	70	35	84
20	74	52	4	85	133	119	64	136
30	108	75	6	120	154	147	87	160
40	130	90	8	141	162	160	104	167
50	151	105	8	155	164	164	112	168
60	160	113	8	161	164	164	117	168

smoothly, and under the same conditions, as does the reaction with penicillamine; thiazolidine derivatives of closely related structure are obtained in both cases [6]. If inhibition by homocysteine (Tables 4-6) is due to an analogous reaction, then it should be expected that homocysteine should condense with pyridoxal phosphate, and that this reaction should proceed readily. In this case, however, the thiazolidine ring cannot be formed; the thiazan ring should be formed instead.

Wriston and Mackenzie [7, 8] have shown that condensation of formaldehyde with homocysteine proceeds smoothly at physiological pH. This reaction may be catalyzed by an enzyme in the organism; it was first observed in liver mitochondrial preparations. The condensation product is 1,3-thiazan-4-carboxylic acid.

An analogous reaction might be expected to take place between homocysteine and pyridoxal phosphate or pyridoxal. We found that the pale yellow color of pyridoxal phosphate solutions fades rapidly after addition of homocysteine. Measurement of the absorption spectra of such systems showed that the maximum at 388 mμ, characteristic of pyridoxal phosphate, had disappeared, and that a new maximum had appeared at 330 mμ

TABLE 4. Effect of Addition of L-Cysteine and DL-Homocysteine on Decarboxylation of L-lysine by B. cadaveris. Phosphate buffer, pH 6.0. Volume of CO_2 evolved, in μl

Reaction time, min	Cysteine content of system, μmoles			Homocysteine content of system, μmoles			Control
	0.25	2.5	25	0.25	2.5	25	
10	43	37	19	40	32	30	42
20	84	70	42	81	70	57	86
30	119	97	65	113	104	81	118
40	143	131	84	137	124	93	140
50	160	143	99	151	144	108	154
60	167	157	112	158	151	119	163

(Fig. 1). Since homocysteine does not absorb in this region of the spectrum, it follows that the new absorption band must be due to the formation of some new product. In order to confirm this supposition we effected a non-enzymic condensation of pyridoxal with homocysteine, and examined some of the properties of the condensation product (thiazancarboxylic acid).

Synthesis of 2-(3-hydroxy-2-methyl-5-hydroxymethylpyridyl-4)-1,3-thiazan-4- carboxylic acid (I). The synthesis of this substance was effected by the same method as that used by Wriston and Mackenzie [8] for the condensation of homocysteine with formaldehyde, with certain modifications. As this synthesis has not been described in the literature, we shall give a somewhat more detailed account of it.

We dissolved 248 mg of DL-homocysteine in 3 ml of distilled water, filtered, adjusted the pH of the filtrate to ~8.0 with NaOH, and transferred it to a stoppered tube with an inlet and outlet for passing gas. Nitrogen was passed through the solution for 5 min, after which we added a solution of 374 mg of pyridoxal chloride in 2.2 ml of water, at pH 8.0, and passed nitrogen for a further 5 min. The tube was then closed tightly, and was left in the dark at room temperature for 24 hr. The solution was then filtered, and the clear, yellow filtrate was freeze-dried. The residue was the sodium salt of I.

Chromatography of the residue, on Whatman No. 1 paper, developed with 80% phenol, gave two spots (ninhydrin spray): one of R_f 0.30 (evidently homocystine), and the other of R_f 0.89, these spots being of approximately equal density. Under the same conditions, homocysteine had an R_f of 0.63

The lyophilized residue was converted into the hydrochloride. This was effected by dissolving 450 mg of residue in 10 ml of distilled water, and passing the solution through a Dowex -1 column, in the PH form. Both the solution and the column were cooled in ice-water. The adsorbed substance was eluted from the column with 0.05 N HCl, the eluate (~100 ml) was evaporated to dryness by lyophilization, and the residue was twice recrystallized from methanol-ether. We obtained 50 mg of crystalline product, decomposing at 152° (uncorrected).

TABLE 5. Effect of Addition of L-Cysteine and of L-Monocysteine on Decarboxylation of Amino Acids by Enzymes from Various Microorganisms. Acetate buffer, pH 5.6. Volume of CO_2 evolved, in μl

Reaction time, min	Cysteine content of system, μmoles			Homocysteine content of system, μmoles			Control	Reaction time, min	Cysteine content of system, μmoles			Homocysteine content of system, μmoles			Control
	0,25	2,5	25	0,25	2,5	25			0,25	2,5	25	0,25	2,5	25	
	Decarboxylation of L-glutamic acid by Cl. welchii SR-12								Decarboxylation of L-aspartic acid by Mycobacterium sp. n.						
10	30	22	18	36	32	15	35	10	148	140	113	144	147	92	146
20	51	39	31	63	56	28	69	20	172	165	158	157	174	171	173
30	73	54	36	85	76	37	84	30	178	173	167	161	177	180	180
40	91	66	48	107	99	47	104	40	176	172	167	162	178	180	180
50	107	78	57	119	104	52	116	50	176	172	166	163	180	180	180
60	122	87	65	134	119	61	134								
	Decarboxylation of L-arginine by E. coli								Decarboxylation of L-histidine by Micrococcus sp. n.						
10	69	68	57	67	59	40	74	10	61	62	49	56	60	52	58
20	115	116	93	116	102	77	127	20	106	106	88	102	103	98	105
30	151	153	134	147	137	109	153	30	133	136	117	125	131	124	135
40	160	163	152	154	148	122	157	40	148	151	135	144	148	140	153
50	162	170	162	156	155	135	168	50	164	169	152	153	156	151	164
60	166	170	166	158	159	142	175	60	180	182	159	160	162	154	186

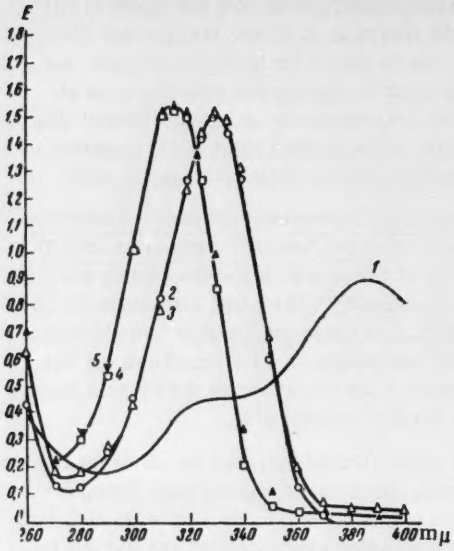


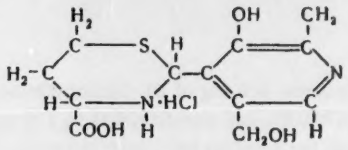
Fig. 1. Ultraviolet absorption spectra in 0.1 M phosphate buffer, pH 6.0. 1) PP (pyridoxal phosphate), 0.20 μmoles/ml; 2) 2-(3-hydroxy-2-methyl-5-hydroxymethylpyridyl-4)-1,3-thiazan-4-carboxylic acid, 0.20 μmoles/ml; 3) PP 0.20 μmoles/ml + HC (homocysteine) 0.63 μmoles/ml, two hr after mixing the solutions; 4) pyridoxal chloride 0.20 μmoles/ml; 5) the same as 4, + HC 0.63 μmoles/ml, two hr after mixing the solutions.

Chromatography under the above conditions gave a single spot, of R_f 0.89. The nitroprusside reaction, in the presence of $NaHCO_3$, was negative.

Elementary analysis of the product gave the following results:

Calculated for $C_{12}H_{16}O_4N_2S \cdot HCl$: C 44.92; H 5.34; N 8.73;
Found: C 44.93; H 5.51; N 8.61.

The results of analysis are in accordance with the conclusion that the substance is the condensation product of pyridoxal with homocysteine, of the following structure:



2-(3-Hydroxy-2-methyl-5-hydroxymethylpyridyl-4)-1,3-thiazan-4-carboxylic acid monohydrochloride (I).

Absorption spectra. The absorption spectra were examined over the interval 270-400 mμ, using a SF-4 spectrophotometer; the substances were dissolved in 0.1 M phosphate buffer of pH 6.0. The following were examined; 1) pyridoxal chloride; 2) a mixture of pyridoxal and homocysteine; 3) a mixture of pyridoxal phosphate and homocysteine; 4) the thiazancarboxylic acid derivative (I) synthesized by us from pyridoxal and homocysteine. The solutions were kept at room temperature for 2 hr before

TABLE 6. Inhibition of Decarboxylase Activity by DL-Penicillamine, L-Cysteine, and DL-Homocysteine, during 10 Minutes, as % of control (without inhibitor)

Decarboxylase	Acetate buffer, pH	Penicillamine, μmoles			Cysteine, μmoles			Homocysteine, μmoles		
		0.25	2.5	2.5	0.25	2.5	25	0.25	2.5	25
B. cadaveris (lysine)	6.0 *	0	5.6	52.8	0	11.9	54.8	4.8	23.8	28.6
E.coli (arginine)	5.6	8.4	16,7	58.4	7.0	8.0	23.0	10.0	20.0	46.0
S. faecalis (tyrosine)	4.5	0	0	45.0	0	5	20.0	0	10.0	25.0
Cl. welchii SR-12 (glutamic acid)	5.6	6.0	16.2	22.3	14.3	37.0	48.6	0	8.6	57.0
Mycobacterium sp. n. (aspartic acid)	5.6	1.0	3.4	15.0	0	4.1	22.6	1.4	0	37.8
Micrococcus sp. n. (histidine)	5.6	0	7.5	35.8	0	0	15.6	3.5	0	10.4

* Phosphate buffer.

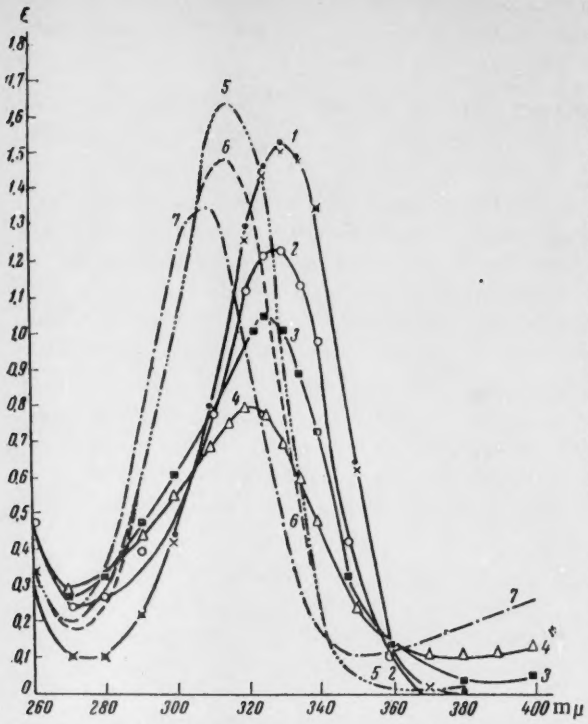


Fig. 2. Ultraviolet absorption spectra of 2-(3-hydroxy-2-methyl-5-hydroxymethylpyridyl-4)-1,3-thiazan-4-carboxylic acid and of pyridoxal chloride. The systems contained 0.10 μmoles of substance in 0.1 M phosphate buffer of different pH: 1) 4.5, 5.0, 6.0; 2) 7.0; 3) 8.0; 4) 9.0, for 2-(3-hydroxy-2-methyl-5-hydroxymethyl-pyridyl-4)-1,3-thiazan-4-carboxylic acid; 5) 6.0, 7.0; 6) 8.0; 7) 9.0, for pyridoxal chloride.

being placed in the spectrophotometer. The results are presented in Fig. 1.

Our compound I has an absorption maximum at 330 mμ (curve 2), as does also the substance rapidly formed in solutions containing pyridoxal phosphate and homocysteine (curve 3). It is of special interest that the phosphate is much more reactive than is free pyridoxal; it condenses with homocysteine as soon as the solutions are mixed, and the reaction proceeds at high velocity at pH 6.0. Under such conditions, pyridoxal does not react with homocysteine. Condensation takes place only at higher pH (~ 8.0), and it proceeds much more slowly. Comparison of the absorption spectra thus shows that the product formed in mixtures of pyridoxal phosphate and homocysteine solutions is identical with the thiazancarboxylic acid derivative I synthesized by us.

We next examined the UV absorption spectra of our compound I at pH 4.5, 5.0, 6.0, 7.0, 8.0, and 9.0, with the object of investigating its stability. The results are presented in Fig. 2. The spectra were identical, with the same absorption maximum at 330 mμ, for solutions of pH 4.5, 5.0, 6.0, and 7.0. At pH 8.0 and 9.0 the optical density was much smaller, and the whole spectrum was shifted somewhat towards the short wave end.

Reactivation by addition of pyridoxal phosphate. For the further elucidation of the mechanism of inhibition by homocysteine we examined the effect of adding pyridoxal phosphate to systems in which decarboxylation had been inhibited by homocysteine.

The experiments were performed with acetone powders of E. coli and B. cadaveris, in a Warburg apparatus at 30°. Two experimental systems were set up, each containing an identical suspension of acetone powder in 2.5 ml of buffer solution, in the main compartment of the Warburg flask, and homocysteine was added to each. The systems were incubated for 15 min, after which amino acid dissolved in 0.5 ml of buffer was added from the side-tube of one flask, and amino acid in 0.25 ml of buffer from one side-tube, and pyridoxal phosphate in 0.25 ml of buffer from the second side-tube

TABLE 7. Reactivation of Decarboxylase Activity of B. cadaveris and E. coli by Addition of Pyridoxal Phosphate (PP) after Incubation for 15 Minutes with Homocysteine. The systems contained 8.3 μmoles of arginine or lysine, and 25 μmoles of PP. Results expressed as μl of CO_2

Reaction time, min	B. cadaveris + L-lysine, phosphate buffer, pH 6.0			E. coli + L-arginine, acetate buffer, pH 5.6			
	control	DL-homocysteine		control	control + PP	DL-homocysteine	
		without PP	in presence of PP			without PP	in presence of PP
10	137	65	95	131	136	56	128
15	149	93	150	151	168	75	163
20	150	108	162	160	177	90	179
30	153	121	167	170	185	118	187
40	153	127	169	179	185	132	187

of the other flask; the two control systems differed from the experimental ones in that one did not contain homocysteine, while the other contained neither homocysteine nor pyridoxal phosphate. The rates of decarboxylation were measured in the usual way. The results are presented in Table 7.

Our results show that pyridoxal phosphate completely abolishes inhibition due to homocysteine.

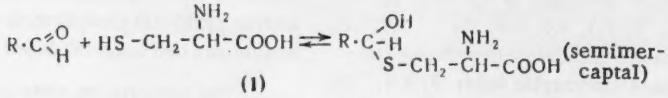
DISCUSSION OF RESULTS

Taken in conjunction with other published results, our findings permit of the conclusion that inhibition of pyridoxal phosphate enzyme systems by penicillamine, cysteine, or homocysteine is due to combination of pyridoxal phosphate with these substances. This involves condensation of the aldehyde group of the pyridoxal coenzyme, to yield catalytically inactive products. The reaction between the sulfhydryl and the amino group of the inhibitor and the aldehyde group of the coenzyme is one of condensation, leading to the formation of a five-membered thiazolidine ring (with penicillamine and with cysteine), or of a six-membered thiazan ring (with homocysteine).

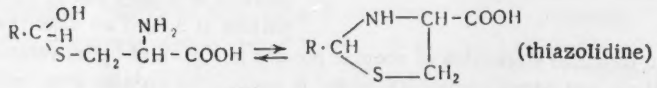
At a concentration of 0.83×10^{-2} M (25 μmoles in the system), penicillamine, cysteine, and homocysteine powerfully inhibit enzymic decarboxylation of amino acids, the reaction rate falling in many cases to 60% of that in the control systems. The inhibitory effect was much smaller at medium and low concentrations of hibitor, being altogether absent in some cases; we were unable to discern any regularities in the behavior of the systems at such concentrations of inhibitor.

Cysteine and homocysteine differ from penicillamine in being widely distributed in living organisms. It has been shown in our laboratory that cysteine also inhibits transamination reactions. It is quite probable that cysteine and homocysteine may, under normal physiological conditions, fulfill a certain regulatory role, by affecting the rates of some biochemical reactions. This would apply in the first place to enzymic reactions involving pyridoxal phosphate as coenzyme.

The reaction of aldehydes with cysteine, to yield thiazolidine derivatives, probably proceeds in two stages [9]:



(I)



(II)

The equilibrium is so far displaced to the right that SH groups cannot usually be detected. However, a number of oxidants, such as hydrogen peroxide or iodine, readily open the thiazolidine ring, giving cystine and aldehyde in almost quantitative yield [12]. It might also be supposed that utilization of aldehyde in other reactions would favor the reverse reaction, from right to left. Thiazolidinecarboxylic acid, formed from cysteine and formaldehyde, readily undergoes oxidation in liver mitochondria, to yield N-formylcysteine [13], which is rapidly further metabolized in liver homogenates. Moreover, thiazolidinecarboxylic acid is as good a source of dietary cystine as is cystine itself [8].

Thiazancarboxylic acid is differently metabolized. In this case, too, condensation of homocysteine with aldehyde probably involves two stage – the semimercaptal is first formed, and is then cyclized to thiazancarboxylic acid. Mackenzie and Wriston [8] have shown that L-thiazancarboxylic acid (synthesized from homocysteine and formaldehyde) is much less biochemically reactive than its lower homolog – thiazolidinecarboxylic acid. L-Thiazancarboxylic acid is not appreciable oxidized in liver homogenates, and it cannot replace cystine in the diet [8]. It may hence be supposed that the products of condensation of homocysteine with aldehydes are not further metabolized, and in this respect the reaction is an irreversible one. It should, however, be borne in mind that the biochemical reactivity of the derivatives is not conditioned only by the fact that they belong to the

thiazolidine or the thiazan series. In contrast to the above findings, thiazolidinecarboxylic acid, produced by the condensation of L-penicillamine with pyridoxal, is not further metabolized, but is excreted unchanged. Administration of L-penicillamine to rats is followed by a sharp rise in urinary vitamin B_6 excretion, and by excretion of large amounts of xanthurenic acid following tryptophan loading [1]. The most important factor determining the biochemical reactivity of different thiazolidine and thiazan derivatives appears to be the availability of enzyme systems capable of making metabolic use of these substances.

Inactivation of enzyme systems involving pyridoxal phosphate as coenzyme by cysteine or homocysteine is a particular case of a widely distributed and important mechanism for regulating the velocity of metabolic processes by means of substance possessing a sulfhydryl and an amino group in their molecule. Living organisms possess a wide assortment of substances containing carbonyl groups (aldehydes or ketones), and which form part of enzymes, hormones, or intermediary metabolites; these substances can enter into condensation reactions with homocysteine, cysteine, or with other substances not usually encountered in Nature, containing a sulfhydryl and an amino group. Such reactions can reversibly or irreversibly exclude these substances from their metabolic pathways, and in this way exert a regulatory effect on the metabolic rate. Thiazolidine derivatives, derived from readily proceeding condensation reactions between cysteine and formaldehyde, pyruvic acid [9], carbohydrates [10], 3-ketosteroids [11], and other products, have been described.

It should also be borne in mind that SH-compounds, including cysteine and homocysteine, can, by condensation with substance possessing carbonyl groups, yield semimercaptals and mercaptals, which can function either as intermediary metabolites or as carriers in transport systems.

SUMMARY

DL-Penicillamine, cysteine, and homocysteine cause considerable inhibition of the reaction of decarboxylation of amino acids by microbial enzyme systems.

The inhibitory effect is due to condensation of pyridoxal phosphate with penicillamine, cysteine, or homocysteine, to yield thiazolidine derivative with the first two inhibitors, and thiazan derivatives with the last. 2-(3-Hydroxy-2-methyl-5-hydroxymethylpyridyl-4)-1,3-thiazan-4-carboxylic acid has been synthesized by condensation of pyridoxal with homocysteine.

Inhibition due to the presence of homocysteine in an enzyme system can be reversed by addition of pyridoxal phosphate.

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A METHOD FOR VITAMIN D ASSAY IN ANIMAL TISSUES

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The few leterature data concerning the use of paper chromatography for vitamin D assay in animal tissues can be found in the works of Kodicek [1,2]. The methods we have developed for separating and determining D provitamins and vitamins [3, 4] allow an approach somewhat differing from that of the above author to analysis of animal tissues characterized by a low vitamin D content.

The first stage in our development of this method was reported at a symposium on vitamins [5]. The present article presents a complete description of the method, which we have already employed to study certain questions of the biochemistry of rachitis and D-hypervitaminosis [6]. The assay process includes: alkali hydrolysis of the tissue; separation of sterols with the aid of digitonin; removal of vitamin A, carotene and other polyene compounds on an Al_2O_3 column; the use of paper chromatography to purify the vitamin D of the sterols not removed by digitonin and determination of vitamin D by the reaction with $SbCl_3$.

By this method, 3-4 μg vitamin D can be assayed; when determining the vitamin D content of animals' blood, therefore, the minimal quantity of blood required for analysis is 10-20 ml from animals receiving vitamin D, 20-30 ml from normal (non-rachitic animals and 40 ml or more from animals with rachitis.

Two main stages comprise this method: chromatography on an Al_2O_3 column and paper chromatography. Vitamin D (a solution of pure D_2 or D_3) is 96-98% recovered with the use of Al_2O_3 (Table 1). Testing various solvents and conditions of vitamin D extraction from a spot on paper showed that total recovery was not effected. Considerable variations from chromatogram to chromatogram were observed with any of the solvents, but the recovery in the separate chromatograms was complerely uniform with little variation. The data in Table 2 on the recovery of vitamin D from a single chromatogram is an example of this. In the first case, the spots were applied in a CO_2 current, in the second, in an air current.

If increasing amounts of vitamin D are applied to the paper at three points, and the values obtained after extraction and quantitative determination are plotted on a graph, the points representing the vitamin recovered should be arranged in a system of coordinates in a straight line. Therefore, regardless of the recovery, the true content of the vitamin in the experimental sample can be computed according to the standard curve for the pure vitamin. The application of this method to analysis of animal tissues is described below.

METHOD

Preparation of Tissues for Analysis: Tissue is taken in batches large enough for the final extract used for paper chromatography to contain at least 3-4 μg vitamin D. To each such batch – 10 ml blood serum, for example – 20 ml ethanol, then 5 ml of a 60% aqueous solution of KOH are added, and hydrolysis is conducted for one hour with a reflux condenser on a boiling water bath. After hydrolysis and cooling, the mixture is diluted with twice its volume of water and extracted four times with 20 ml peroxide-free ether. The ether extract is washed with water until the wash waters give a neutral reaction and dried with anhydrous Na_2SO_4, and the ether is distilled off to dryness.

TABLE 1. Vitamin D Recovery with 500 mg Al_2O_3, Elution by 40 ml Petroleum Ether with a Boiling Point of 50-70°

Vitamins	Vitamin used in international units	Vitamin Recovered	
		units	%
A	400	0	0
D_2	400	391	97.3
D_2	200	196	98.0
D_3	400	392	98.0
D_3	200	192	96.0

TABLE 2. Vitamin D_2 Recovery after Paper Chromatography; Amount of Vitamin Shown in International Units and, in Parentheses, in Percent

Vitamin D_2 applied	Vitamin D_2 recovered	
	without CO_2	in CO_2 current
100	50 (50)	80 (80)
200	94 (47)	164 (82)
400	216 (54)	312 (78)

The residue is dissolved in 5 ml ethanol, and the sterols are precipitated by heating on a water bath to 60-70° and the addition of 5 ml of a 1% alcoholic solution of digitonin. The mixture is kept on the water bath for four to five minutes: the alcohol is diluted with water to 72% of its volume, cooled to room temperature and then put in a refrigerator for 20-30 minutes.

After removal of the digitonide preciptate, the filtrate is diluted twice with water, and the lipoids are converted into an ether extract. Water is used to wash the alcohol and surplus digitonin from the extract, which is then dried and the ether distilled off. The residue in the flask is dissolved in 1 ml petroleum ether (boiling point, 50-70°) and purified of vitamin A, carotenoids and other admixtures by being passed through a 500 mg Al_2O_3 (the Al_2O_3 used for chromatography; second grade of activity) column, 0.6-0.7 cm in diameter and 20-22 cm in height. The column is washed with 40 ml petroleum ether. The ether is distilled off, and the residue is transferred in small amounts of sulfuric ether to a narrow test tube, the ether being evaporated each time on a water bath. After the unsaponifiable fraction is transferred to the test tube, 0.1-0.2 ml methanol is added to the latter. The resulting solution of the unsaponifiable fraction is applied to the paper.

Preparation of Paper for Chromatography: Brand "B" Leningrad chromatographic paper is cut into strips measuring 14 × 60 cm, rolled loosely into a tube and put in 10% HCl for 20 hours. Then the acid is poured off, and the paper is washed with distilled water (heated to 60-70°) until a neutral reaction is obtained and dried at room temperature. Then, without being rolled up, each sheet is separately moistened with 5% $Ca(H_2PO_4)_2$ in H_2O by being passed through the solution. The sheets of paper are dried slighly in a draft and then passed through 2 N NH_4OH. The sheets are again dried slightly, rolled up together, put into a glass, washed free of ammonia with distilled water and dried at room temperature. Before use, the paper is put into a chamber overnight and washed with a descending stream of a mixture consisting of petroleum ether and acetone (9:1), 30−35 ml. Then the paper is dried in a draft and cut into halves measuring 14 × 30 cm.

Paper Chromatography: A standard solution of vitamin D is applied in increasing amounts at three points on the 14 × 30 sheet of paper: 100, 200 and 300 international units and one spot of the experimental sample. If a larger chamber and paper are used, several spots of experimental samples can be applied.

The solution are applied in a CO_2 current to prevent oxidation of the vitamin by the air oxygen. The following procedure is used for this purpose. A funnel 3 cm in diameter is attached to a water-jet pump through a Bunsen flask, and a second funnel of equal diameter is attached to a CO_2 source. The portion of the paper to which the spot is to be applied is placed between the funnels so that the spot, shielded by the connected funnels, is quickly dried in the CO_2 current. After the spots have been applied, the paper is put into a chamber, and chromatography is performed by the descending method, using the active phase: petroleum ether − acetone, 9:1. Chromatography is stopped after the solvent front has advanced 20-25 cm (for which 60-80 minutes are required).

Vitamin D Assay: The chromatogram is put into a jar with CO_2 and examined, after evaporation of the solvent, in an ultrachemiscope. Dark spots are apparent in the vitamin D localization zone if the spot contains at least 150 international units of the vitamin; when the vitamin content is 100 international units, the spot is not always detected. The largest of the vitamin D spots is cut out, and the others according to its measure, so that the weight of each spot is the same. In the case of low concentrations of the vitamin, when the spots are not apparent in ultraviolet light, they are cut out according to the position of the visible spots.

The piece of paper are weighed and cut into small pieces, 1 ml chloroform is added, and the test tubes are stoppered and allowed to stand for 30 minutes, after which 4 drops of acetic anhydride and 6 ml of a 23% $SbCl_3$ solution in chloroform are added to each test tube. After three minutes, the solution is poured off into the cuvette

TABLE 3. Biological Testing of the Method

Specimens under analysis	vitamin D found in I.U. per 1 ml blood or 1 g tissue	
	by biological tests	by chemical method
Oil solution of unsaponifiable fraction of horse's blood	2	2
Rabbit's blood	6.5	7.2
Rabbit's liver	2.0	2.5
Chicken blood serum	5	10

of a photoelectric colorimeter with a working length of 20 mm and the optical density at 500 mμ is measured exactly four minutes from the time the $SbCl_3$ was added. Pure chloroform is used as the compensating solution.

A standard calibrated curve is constructed for the given chromatogram from the values of optical density obtained for pure vitamin D. The vitamin D content of the experimental specimen is calculated from its content in the experimental spot (according to the determination along the standard curve), the quantity of the final alcoholic extract used in the chromatography and the amount of the material used for analysis.

BIOLOGICAL TESTING OF THE METHOD

An oil solution of the unsaponifiable fraction of horse's blood (1 ml per 17 ml blood), the heat-preserved blood and liver of vitaminized rabbits and frozen fresh blood serum from chickens fed the poultry food manufactured by the "Kuntsevo" factory. Except for the results of the experiments with the chicken blood serum, the data from the biological tests matched the results obtained by the method described above (Table 3).

The divergent results obtained by the two methods for the chicken blood serum could have been caused by oxidation of the vitamin D by the blood hemins, the oxidizing effect of which on D provitamins, vitamin A and unsaturated fatty acids is known from the literature [7-10]. The serum we used contained remnants of hemins. To prove the possibility of the destructive action of the blood hemins, we added 800 international units of vitamin D_2 to 10 ml whole rat's blood combined with 10 mg NaF. The mixture was allowed to stand under toluene in an incubator at 37° for 17 hrours, after which we found only 480 international units, i.e., 60% of the vitamin D had been oxidized.

APPLICATION OF THE METHOD

We studied vitamin D distribution in rabbit's tissues. The animals were given vitamin D per os for 40 days, each animal receiving a total of 23,000 international units of the vitamin. Before the experiment began, the average blood sample from three experimental rabbits contained 0.6 international units per 1 ml, at the end of the experiment, the content was 7.2 international units. The tissue richest in vitamin D content was that of the adrenal glands (28 internation units per 1 g of raw weight); next in order of vitamin D content were: the blood (7.2), lungs (6.1), liver (2.5), kidneys (2) and heart (1.8), the smallest vitamin D content being found in the muscles (0.84) and intestines (0.62). The results we obtained on vitamin D distribution in animal tissues agree with the literature data [5, 12-14].

The method has also been applied in zootechnical experiments* on calves and piglets. The calves were kept under identical conditions except for the fact that one group received hay dried in the sun, while the other received hay dried in the shade. The vitamin D content in the calves of the first group equalled 9-10 international units per ml blood, while vitamin D could not always be found in the blood of the calves of the second group, and clear signs of rachitis were observed in the animals. Thomas and Moore [11] have described analogous experiments with similar results.

In the experiments with piglets, one group was given vitamin D_2 for two months in the form of irradiated dry yeast in a dose of 15 international units a day per 1 kg animal weight, while the second group served as the control. At the end of the experiment, the vitamin D content in the animals of the first group was 9-11 international units per ml in the blood serum and 19-30 international units per g in the liver, but only 4-6 international units in the blood and liver of the animals in the control group. The data cited suggest that this method can be used in zootechnical investigations.

*The experiments were conducted by E. A. Nestorova at the All-Union Institute for Scientific Research in Animal Husbandry.

SUMMARY

A method is described for vitamin D assay in animal tissues, consisting in alkali hydrolysis of the tissue, removal of sterols by digitonin, separation of vitamin A and other polyene compounds on an Al_2O_3 column and final purification of vitamin D of admixtures by paper chromatography. Determination of the vitamin extracted from the paper is carried out by means of the reaction with $SbCl_3$. The method gives easily reproducible results. Examples of the method's use are cited.

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THE POSSIBLE TRANSFORMATION OF BLOOD PLASMA ALBUMIN AND γ-GLOBULIN INTO α- AND β-GLOBULINS

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The possible transformation of blood plasma albumin into globulin has been demonstrated by isotopic methods [1]. It was not evident from these experiments, however, whether the transformation occurs at the second or third order level of structure or whether it is related to the breakdown of the protein molecule into amino acids. In this communication, further demonstration is given in favor of the hypothesis we have expressed [2, 3], that some part of the α- and β-globulins are products of the transformation of albumin and γ-globulin without an extensive breakdown of the latter.

At the present time, the generally held opinion is considered to be, that α- and β-globulin fulfill a transport function [4, 5]. In this manner some of the metabolites are transferred in the form of protein complexes. The physical chemical properties of the complexes must differ from the properties of the starting substances. In this connection the question arises: why are the most diverse protein complexes (lipid, glucide, complexes with metals, etc). found in the plasma in one or two electrophoretic fractions. There are two possible explanations for this phenomenon.

Specific synthetic processes occur in the cells as a result of which protein metabolite pairs are always selected so that complexes are formed with primarily a single mobility. This type of coupling can have some physiological significance, if it is assumed that under the conditions of transfer of the complexes in the organism a definite value of their effective charge is necessary.

The second possibility is based on the assumption that the phenomenon of electrophoretic homogenization EH [6-8] is related to all processes of complex formation. In this case changes similar to denaturation occur in the protein and as a consequence, different protein complexes acquire almost a single electrophoretic mobility. Apparently this is only possible for those proteins which do not differ too greatly in their amino acid composition. Thus, it is assumed that many processes of complex formation are related to the physical chemical process, EH. A complex may be formed independently as a result of a simple chemical interaction or this process is catalyzed by enzymes; it is assumed, that it can be accompanied by EH. It can be supposed that EH accompanies the process of complex formation in all those cases, when the share of nonprotein component in the complex is large and the second and third order protein structures become unstable.*

The connection between complex formation and the destruction of the native protein was examined by Flory in its thermodynamic aspect [9]. On the basis of their data, obtained with fibrillar proteins, it may be

*Destruction of the secondary and tertiary structures msut take place in all those cases, when as a result of complex formation the stability of these structures is lowered, and when the reaction takes place in open solution, where the arrangement of the reacting molecules is accidental. This consideration is not related to the case in which addition of non-protein components takes place on the "matrix" surface which determines the macrostructure of the complex. There is sufficient basis to assume, that the reaction of complex formation (enzymatic or non-enzymatic) takes place in an open solution.

assumed that EH of plasma proteins is related to the phase transfer of native "crystalline protein" (regularly oriented) into "amorphous" (statistical coils).

The question of whether electrophoretic homogenization definitely is considered a denaturation or whether change in the mobility in this case is similar to isomerization, described by Aoki and Foster [10]* Saifer and Corey [11], is impossible of final solution.

If we accept the opinion developed here, then it can be assumed that the albumin complexes according to mobility, appear to be in the α-globulin group, and the γ-globulin complexes in the β-globulin group because of disorientation of the native structure [6, 8]. It does not follow from this that all proteins from the α-and β-globulin groups are unchanged forms of albumin and γ-globulin, since specific proteins, enzymes, etc. are found in this group.

It is possible, that in an organism in a pathologic state of the process of transformation of albumin and γ-globulin into α- and β-globulins is increased (for example, there are the C-reactive or paraprotein proteins).

Two basic questions are examined in the light of the statements made above: 1) what happens to the EH reaction on chemical treatment of the protein: 2) does the EH phenomenon occur in experiments on living models.

METHODS

In Vitro Experiments

The investigations were made on whole bull blood serum and bull serum protein was also used—albumin and γ-globulin. On the basis of previous investigations [13] it can be assumed that on protein denaturation analagous phenomena occur in the blood sera of other animals.

Preparations of separate protein fractions were obtained from bull serum by fractionation of the serum by ammonium sulfate precipitation, as described earlier [13].

The γ-globulin preparation for the most part was electrophoretically pure, although sometimes it contained a small impurity of α- and β-globulin (up to 5%). The albumin preparation contained up to 15-20% of α- and β-globulin. In order to obtain the highest purity of albumin (albumin content 92-95%), three reprecipitations of the albumin were accomplished from very small amounts of the original preparation. The impurities in this preparation were 5-8%. Great difficulty in obtaining albumin preparations with minimum globulin impurities have been noted by other authors [12]. The basic experiments were repeated with such purified preparations for control. No significant differences in results of treatment between the purified and nonpurified preparations of albumin were noted.

Whole serum proteins were treated at room temperature with the following reagents:

Formaldehyde—0.1 ml undiluted solution of formalin in 10 ml serum; one sample was kept 2 hours at 37°, the second—20 hours at 37°, the third—2 hours at 60°.

K thiocyanate—1,3 and 7 g per 10 ml serum, 2 hours.

Cyclohexylamine—1, 2, and 3 g per 10 ml serum, 3 hours

Urethan—0.5, 1, and 2 g per 10 ml serum, 2 hours.

Na salicylate—1, 1.5, and 2 g per 10 ml serum, 2 hours.

Urea—3, 5, and 7 g per 10 ml serum, 2 hours.

p-Aminosalicylic acid (PASA)—1, 3, and 5 g per 10 ml serum, 28 hours.

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*Change in the molecular dimensions and form of the protein, in conformity with the equation of Henry [25] and Linderstrom-Lang [26], causes a change in the electrophoretic mobility and ionization constants of ionized groups. In the first approximation this effect is independent of a possible increase in dimensions of the protein molecule as a result of the transfer oriented → unoriented (denaturation) or whether "reverse expansion" in the acid medium occurs [10], or the radii of the molecules change for some other reason.

Ammonia – 5 and 10 ml aqueous solution per 10 ml serum (at pH 11.3-11.5), 2 hours.

If the treatment of the protein preparations was carried out in acid or alkaline media, then the necessary pH was obtained by the addition of 0.1 N NaOH or 0.1 N HCl. The experimental samples were then submitted to treatment, and the control samples were kept in the refrigerator. According to the lapse of time of treatment the solutions were dialyzed 10-12 hours against flowing tap water, then against 1% NaCl for approximately half a day. Electrophoresis was carried out by the method of moving boundaries with optical registration by a method described previously [14]. A veronal buffer, pH 8.6 and μ0.1, was used for electrophoresis. Calculation of the ratio of the protein fractions and calculation of the mobility of the separate fractions according to the electrophoretogram were carried out by standard methods using a mechanical coulomb meter [24].

EXPERIMENTS WITH SURVIVING ORGANS

Surviving rabbit heart and kidney perfused with serum were used for experiments. Blood was taken from the outer ear vein: serum was obtained by the standard method and kept not longer than 2 days at 2-4°.

Before removal of the heart, the animal was stunned, the chest cage was opened and the heart was quickly isolated. A cannula was inserted, in a section of the aorta, through which the heart was washed with warm Ringer-Locke solution, after which the cannula was connected with a burette filled with the same solution, constantly saturated with oxygen from a KIP-3 apparatus. The perfusion was carried out under a pressure of 70-80 cm from a water column, the temperature of the perfusing liquid was 38°.

In the first 1-2 minutes of perfusion the heart beat arhythmically, after which the heart beat became regular, but somewhat slower. After 5-10 minutes of stable and rhythmic heart beats perfusion with blood serum diluted twice with Ringer-Locke solution was begun.

Another burette of the same height but smaller volume was used for perfusion with serum, which permitted the use of a smaller amount of serum (25-30 ml). The serum was also saturated with oxygen and heated in a thermostat. The perfusion was carried out for one hour, in the course of which the same volume of serum passed through the heart approximately 30 times.

As a control, some experiments were performed by passing serum diluted twice with Ringer-Locke solution through the system used in the experiment (without the heart).

The kidney was taken from a rabbit stunned with a blow, quickly washed and placed in a plexiglass chamber with water heated to 37-38°. Perfusion was carried out through the renal artery; the perfusate freely flowing out of the vein was again used for perfusion.

Experimental nephrosis was induced by repeated intravenous injection into the rabbit every 2-3 days (11 injections total) of 0.4 mg/kg uranium nitrate. The serum before and after perfusion was dialyzed and investigated by electrophoresis as described above.

RESULTS OF THE INVESTIGATION AND DISCUSSION

In vitro experiments. Changes in the content of the protein fractions of blood serum after treatment with various concentrations of the test reagents are graphically illustrated in Figures 1 and 2. In the first part of each figure data are presented for electrophoretic analysis, in the second part, resutls of the salt fractionation. In Figure 3 electrophoretograms are presented, obtained by analysis of the treated sera. Only a single concentration of each reagent among those tested is given which shows the most characteristic change (the selected concentration is not necessarily the maximum).

It is evident from these data that electrophoretic changes in the serum proteins under the influence of various agents is extremely monotypic and always is expressed by increase in the fractions moving at the rate of α_2- or β_1- globulins. These changes are pronounced for some reagents (urea, PASA, cyclohexylamine, etc.) and only slightly evident for others (urethan, etc.).

According to the data from the salting out procedure, the investigated reagents can be separated into two groups. The majority of agents (formaldehyde, K thiocyanate, cyclohexylamine, urethan and Na salicylate – Figure 1) act on serum proteins similarly to heat [8, 13, 15]. On salt fractionation of the sera treated by these agents the euglobulin protein content increases markedly; it is separated at a low salt concentration.

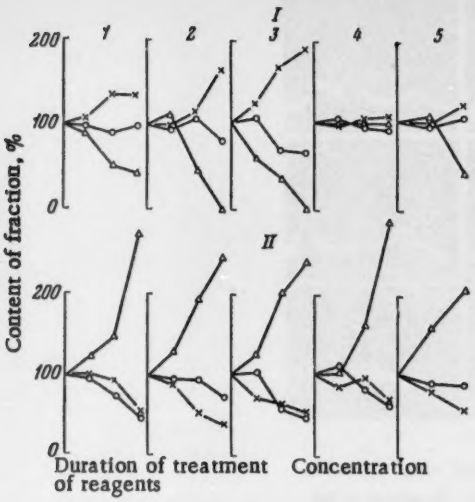

Fig. 1. Change in the content of serum protein fractions after treatment with reagents of the first group. I) By electrophoretic analysis, II) By salt fractionation. o-o-o-, albumin, x-x-x- $\alpha + \beta$ globulins (pseudoglobulins); Δ-Δ-Δ-γ-globulins (euglobulins); 1) formaldehyde, 2 hours at 37°, 20 hours at 37° and 2 hours at 60° 2) K thiocyanate 1, 3, and 7 (g/10 ml; 3) cyclohexylamine, 1, 2, and 3 g/10 ml; 4) urethan 0.5, 1 and 2 g/10 ml; 5) Na salycylate 1 and 1.5 g/10 ml.

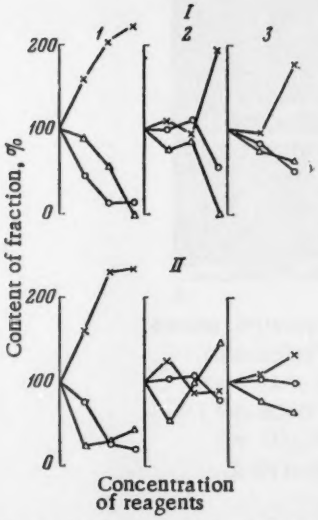

Fig. 2. Change in content of serum protein fractions after treatment with reagents of the second group. 1) Urea, 3.5 and 7 g/10 ml; 2) PASA, 1.3 and 5 g/10 ml; 3) ammonia, 5 and 10 ml/10 ml. Remaining designations the same as in Figure 1.

Urea, PASA and ammonia can be placed in the second group of agents (Fig. 3). Urea has the most pronounced effect among this group of agents. Treatment with urea in the majority of cases causes an increase in the concentration of proteins salted out by medium salt concentrations, i.e. the pseudoglobulins are increased. Thus, both according to electrophoretic and salting-out data, treatment of serum proteins with urea leads to an increase in the "middle" group of proteins (α-β-globulins by electrophoresis and pseudoglobulins, by salt fractionation.

It is necessary to note that the highest concentration of urea (7 g/ml and sometimes 5 g/10 ml) in approximately one out of three experiments led to an increase in the euglobulin content. The urea concentration of 3 g/10 ml constantly caused an increase in pseudoglobulin.

PASA at fairly low concentration (1 g/10 ml) causes the same changes as urea, although less pronounced. High concentration of PASA increased the euglobulin.

Increased pseudoglobulin after treatment with ammonia was noted in only half of the experiments. The effect of ammonia was conditional on its alkaline properties: addition of NaOH to the same pH (11.3-11.5) showed approximately similar activity in salt fractionation.

Various chemical agents can cause serum proteins in all cases to undergo electrophoretic homogenization, but investigation of salting out can demonstrate an increase either in the euglobulins or in the pseudoglobulins. These data are of definite interest, since it is known that native α- and β-globulins are composed both of pseudoglobulins and of euglobulins. Although in these experiments very high concentrations of reagents are used for the determination of the transfer of protein from one electrophoretic group to the other. The results merit attention, since in the organism reactions of complex formation can be catalyzed by the corresponding enzymes, and hence the presence of a high concentration of non-protein substance at each particular moment is not necessary. In this respect it must be noted that the nonprotein component in natural complexes can attain 70% of the total weight. The substances used are far from being natural nonprotein components of complexes; it may be noted, however, as was shown earlier, that acetaldehyde, butyric aldehyde and some quinones [3] possess similar activity.

The activity of a series of reagents was verified also on isolated albumin and γ-globulin of bull blood serum. The corresponding electrophoretograms are presented in Figures 4 and 5.

Isolated albumin and γ-globulin are changed in a similar manner to the fractions serum, but the degree of these changes varies. Isolated albumin is more stable to the activity of urea, K thiocyanate, formaldehyde and PASA, than the albumin fraction of the whole serum. In neutral

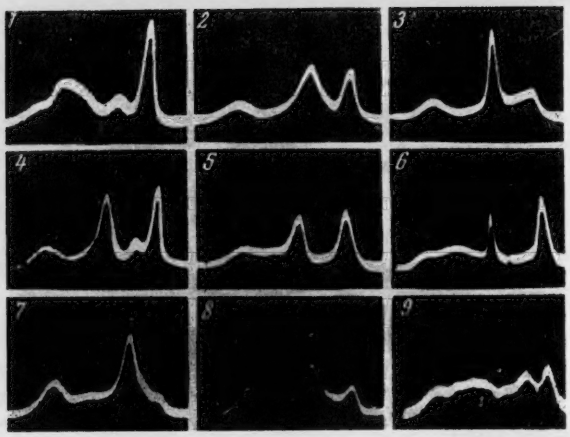

Fig. 3. Electrophoretograms of bull blood serum, treated with various reagents. 1) Original serum; 2) serum after treatment with formaldehyde (0.1 ml 30% solution/10 ml, 20 hours at 37°); 3) K thiocyanate 7 g/10 ml; 4) cyclohexylamine 2 g/10 ml; 5) Na salicylate 1.5 g/10 ml 6) urethan 2 g/10 ml; 7) urea 7 g/10 ml; 8) PASA 5 g/10 ml; 9) ammonia 10 ml/10 ml.

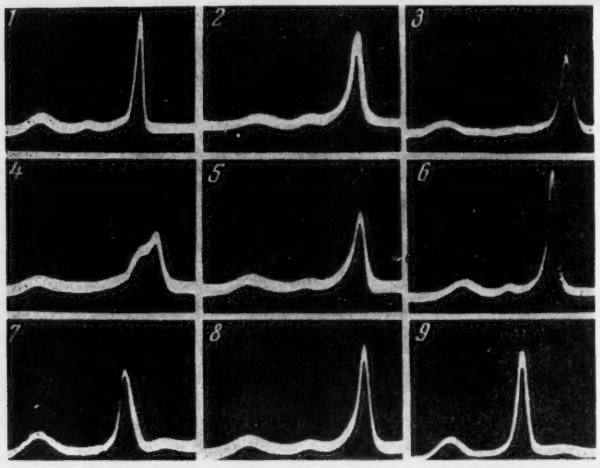

Fig. 4. Electrophoretograms of the albumin preparation, treated with various reagents. 1) Original preparation; 2) preparation, treated with PASA 5 g/10 ml; 3) formaldehyde (0.1 ml 30% solution/10 ml, 20 hours at 37°; 4) solution of NaOH (pH 11) 2 hours; 5) urea 5 g/10 ml; 6) K thiocyanate 7 g/10 ml; 7) urea, 5 g/10 ml at pH 11; 8) urea 5 g/10 ml at pH 2; 9) potassium thiocyanate 7 g/10 ml at pH 11.

medium the investigated reagents increase by only 10-15% the relative amount of α-globulin in the preparation, on the other hand, in general, the electrophoretogram of the original preparation does not change (Fig. 4, curves 2, 3, 5, and 6). Only the action of the reagents in alkaline medium (pH 11) almost completely converts the albumin into protein with the mobility of α-globulin (curve 7 and 9). The OH^- ion converts approximately half of the albumin into protein with the mobility of α-globulin (curve 4). An acid medium does not possess such

TABLE 1. Changes in Composition and Mobility of Separate Fractions of the Albumin Preparation after Treatment with Various Reagents

Experimental conditions	Content of the fraction, relative %			Mobility of the fraction, $cm^2 \cdot sec^{-1} \cdot \beta^{-1} \cdot 10^{-5}$		
	albumin	α-globulin	β-globulin	albumin	α-globulin	β-globulin
Original preparation	85.3	3.6	11.1	7.4	6.3	3.8
PASA 5 g/10 ml	81.0	10.0	9.0	7.4	6.2	4.1
Formaldehyde 0.1 ml/10 ml, 20 hours at 37°	88.4	7.4	4.2	8.1	6.7	4.7
Urea 5 g/10 ml	73.0	14.0	13.0	7.5	6.4	3.8
Potassium thiocyanate 7 g/10 ml	75.6	12.2	12.2	7.4	6.3	3.8
Alkaline medium, pH 11	53.0	47.0	0	7.5	6.7	–
Urea 5 g/10 ml, pH 11	11.3	88.7	0	8.2	5.7	–
Potassium thiocyanate 7 g/10 ml, pH 11	8.1	91.9	0	9.5	5.8	–
Urea, 5 g/10 ml, pH 2	75.6	12.2	12.2	7.4	6.3	3.9

TABLE 2. Changes in the Composition and Mobility of the γ-Globulin Preparation after Treatment with Various Reagents

Experimental conditions	Content of the fraction, relative %		Mobility of the fraction, $cm^2 \cdot sec^{-1} \cdot \beta^{-1} \cdot 10^{-5}$	
	β-globulin	γ-globulin	β-globulin	γ-globulin
Original preparation	0	100.0	–	2.5
Formaldehyde 0.1 ml/10 ml, 20 hours at 37°	78.0	22.0	3.8	2.9
Potassium thiocyanate 7 g/10 ml	87.4	12.6	3.4	2.1
PASA 5 g/10 ml	100.0	0	3.3	–
Urea 5 g/10 ml	0	100.0	–	2.4
Urea 7 g/10 ml	21.1	78.9	3.4	2.5
Alakaline medium, pH 11	100.0	0	6.5	–
Urea 7 g/10 ml, pH 11	100.0	0	3.4	–

marked activity: treatment with urea at pH 2 has little effect on the protein composition of the preparation (curve 8). The specific action of formaldehyde is shown by an increase in mobility of all fractions to approximately the same degree. Treatment with urea and K thiocyanate in alkaline medium leads to increased mobility of the albumin peak of the protein. Values for electrophoretic mobilities and the composition of separate fractions of the albumin preparation after treatment are presented in Table 1.

Isolated γ-globulin in comparison with γ-globulin of the whole serum fraction is more stable to urea; only 20-25% of the γ-globulin converts to protein with the mobility of β_1-globulin at a urea concentration of 7g/10 ml (Figure 5, curves 5 and 6). K thiocyanate and formaldehyde almost completely transform isolated γ-globulin into protein with the mobility of β_1-globulin (curves 3 and 2); PASA brings about 100% transformation (curve 4). The action of urea in alkaline medium (curve 8), as well as alkaline medium alone completely change the mobility of γ-globulin. Changes in the electrophoretic mobility of isolated γ-globulin after treatment are presented in Table 2.

Thus, the isolated albumin is transformed by various chemical reagents into a fraction with decreased mobility (in comparison with the original preparations).

Experiments with surviving organs. Although direct synthesis of natural complexes of the α- and β-globulin type is not possible at this stage, for the purpose of the experiment the method of surviving organs and tissues was used. Earlier Roberts and Kelley [16] using labelled atoms, showed that liver slices can synthesize α-globulin from labelled albumin. However these experiments did not indicate a direct transformation of one

protein into another, as assumed by the authors, since the liver contains proteolytic enzymes, capable of destroying the albumin and thus synthesizing α-globulin from the breakdwon products of albumin. Aside from this, many natural α-globulins are contained in the livers, and hence extraction of liver globulin and contamination of the added albumin could occur, since both proteins are capable of forming complexes. It is also possible that the label is transferred. From these considerations, in order to demonstrate the possible transfer of albumin into α-globulin we decided to use another model: surviving heart. This material was convenient because of the fact that the synthesis of plasma proteins does not occur in heart tissue, apparently, and the natural blood proteins are washed out by previous perfusion with Ringer-Locke solution. On the other hand, the metabolism of substances in this organ is very vigorous and is easily controlled by the intensity of contraction.

It is assumed that metabolites, extracted from the organ in the perfusing liquid, bring about "αβ-globulinization," that is, an increase in α- and β-globulins and decrease in albumin [6]. "αβ-globulinization" was shown to be similar to the effect of EH [8, 13], created by the activity of various substances described in the first part of the present communication.

Previous results of experiments on perfusion of the heart were communicated earlier [6]. New data are presented in this paper as well as results of the necessary control experiments, omitted earlier (Table 3).

From these data it is evident that perfusion of the heart provides statistical proof of an increase in the concentration of α-globulin at the expense of a decrease in albumin concentration, that is, a typical EH effect. Change in the concentration of γ-globulin is not statistically proved, although in a number of cases there is a significant change in this fraction. The control experiments also indicate a change in the composition of serum proteins, but they are opposite in effect to the experiment. We observed an increase in the concentration of albumin at the expense of a decrease in the concentration of globulins, during perfusion through the system not including the heart. Such a result was not expected, although it is known that a similar effect is caused by heating of the serum to a temperature of 50° [13]. It can be assumed that factors causing changes in the proteins appear on filtration of the plasma through the capillary system of the heart. Regretfully it was not possible to set up experiments with dead heart because of the fact that the capillaries of the heart are markedly contracted on death, and the perfused serum is not conducted. In order to clarify this problem we used another model—the surviving kidney. Experiments were established with the healthy organ and "nephrotic" kidney, taken from an animal, which previously (in order to cause nephrosis) had been injected with uranium nitrate. Results of this series of experiments are presented in Table 4.

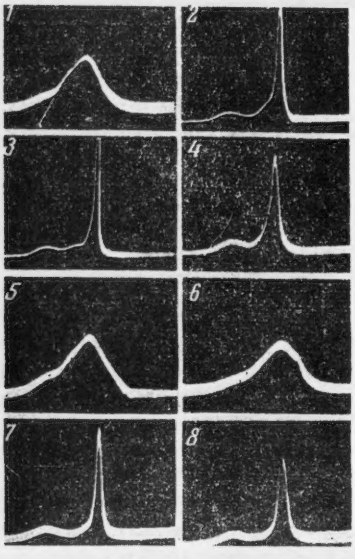

Fig. 5. Electrophoretogram of the γ-globulin preparation, treated with various reagents. 1) Original preparation; 2) preparation teated with formaldehyde (0.1 ml 30% solution/10 ml, 20 hours at 37°); 3) K thiocyanate 7 g/10 ml; 4) PASA 5 g/10 ml; 5) urea 5 g/10 ml; 6) urea 7 g/10 ml; 7) solution of NaOH (pH 11); 2 hours; 8) urea 7 g/10 ml at pH 11.

From the values presented in Table 4 it results that perfusion of the healthy kidney also causes EH, expressed by an increase in the α-globulins at the expense of albumin. The diseased kidney does not possess such ability. This demonstrates that the fact itself of perfusion through the capillary system is not related to changes in the proteins. In this case the reason why the diseased kidney does not cause an EH effect, has secondary significance in comparison with the facts themselves that perfusion does not take place in the organ in these conditions, and that its use as a control is possible. In order to exclude the possibility of the protein being washed out from the organ, analysis of the perfusing liquid was carried out, without added protein (Ringer-Locke solution). This solution was passed through the heart many times in the same quatities and under the same conditions as the serum. The presence of protein in the perfusate was determined by precipitation with sulfosalicylic acid. It was shown that during lengthy perfusion in Ringer-Locke solution a trace of protein was transferred; its concentration could not be registered on electrophoresis. Furthermore, the concentration of proteins in the perfusing serum remained unchanged in all our

TABLE 3. Changes in Blood Serum Proteins in the Process of Perfusion through Isolated Heart. The values presented are the ratio of the mean difference to the original serum ± the mean error.

	number of experiments	albumins	globulins		
			α	β	γ
Perfusion through the system, excluding the heart	5	+2.5±0.8	−1.9+0.4	+0.6±0.62	−0.7±1.0
Perfusion through the system, including the heart	22	−3.9±0.85	+1.5±0.27	+2.1±0.57	+0.3±0.66
Coefficient of significance		4.8	6.8	1.76	0.77
Probability of the difference (level of significance)		>>0.99	>>0.99	insignificant	insignificant
Protein composition of the serum before perfusion (arithmetical mean)	23	60.1	9.3	15.5	15.1

experiments. If it is assumed that protein is washed out, then according to the observed electrophoretic changes, a considerable increase in the total concentration of the protein must be expected. Thus, the experiments described indicate that the very fact of the passage of the protein through the functioning organ causes a phenomenon similar to that described in the first part of the present paper.

The established facts can be explained by denaturation effects, if it is assumed while the heart is functioning substances are excreted into the perfusing liquid which cause the denaturation (or cause EH, if it is considered that both effects differ in mechanism). Such a conclusion is naturally possible in as much as, the washing out of the protein or its injury as a result of passage through the vessels of the organ is excluded as stated above. It is naturally assumed, that similar relationships exist in the living organism; in the opposite case it would have resulted that the existence of a mechanism would have to be postulated which would guard against such changes (for example, preventing a change in the protein or its regeneration). In this respect it must be noted, that at the present time the necessity of serum albumin for oxidative phosphorylation has been established. Albumin is linked to an inhibitor of oxidation, which, possibly, has a quinoid structure [17].

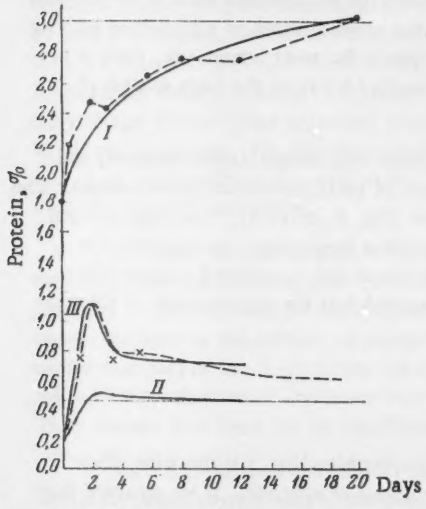

Fig. 6. Comparison of experimental and calculated curves for the dynamics of blood proteins during their regeneration. Dotted line, experimental curve; solid line, calculated curve; I, regeneration of albumin; II and III, regeneration of α- globulin.

Differential equations, describing the interaction of two proteins, associated with a transport function. If α-globulin is a product of transformation of albumin while fulfilling a transport function, then a simple relationship between both proteins must be expected. This relationship can be presented on the basis of calculations, devised by one of us. Let us assume, that the formation of α-globulin proceeds in accordance with the reaction:

$$\text{albumin} + X \rightleftharpoons \alpha\text{-globulin} \qquad (1)$$

Then $C_\alpha / (C_A \cdot C_X) = K$, where $C_{\alpha, A, X}$ are the corresponding concentrations, and K is a constant.

It is known, that metabolism can be regarded as a stationary process, so that the rate of synthesis of X can be regarded as constant (φ_1), and then

$$C_X = \varphi_1 t. \qquad (2)$$

Let us assume, now, that X is constantly removed from the tissue as a result of complex formation with albumin. In this case its concentration no longer is determined by equation 2, and will still depend on the rate of removal of X. In the simplest case the concentration of X can be found from the equation:

$$\frac{dC_X}{dt} = \varphi_1 - KC_A \cdot C_X \qquad (3)$$

TABLE 4. Changes in Blood Serum Proteins in the Process of Perfusion Through the Kidney. The values presented are the ratio of the mean differences, to the starting serum ± the mean error.

Experimental procedure	number of experiments	albumins	globulins		
			α	β	γ
		healthy kidney			
Perfusion through the system, excluding the kidney	5	+2.5±0.8	−1.9±0.4	+0.6±0.62	−0.7±1.0
Perfusion through the system, including the kidney	5	−3.7±1.3	+3.1±0.7	+1.5±0.4	−1.3±0.3
Coefficient of significance		4.0	6.2	1.3	0.6
Probability of the difference (level of significance)		>> 0.99	>> 0.99	insignificant	insignificant
		"nephrotic" kidney			
Perfusion through the system, excluding the kidney	5	+2.5±0.8	−1.9+0.4	+0.6±0.62	−0.7±1.0
Perfusion through the system, including the kidney	5	−0.2±1.3	+0.2±0.7	−1.1±1.0	+1.0±0.3
Coefficient of significance		1.8	2.0	1.3	1.7
Probability of the difference (level of significance)		insignificant	insignificant	insignificant	insignificant

Further, this rate of removal α-globulin from the blood is proportional to its concentration and the constant f. Then its concentration in the blood will be determined by the equation:

$$\frac{dC_\alpha}{dt} = KC_A \cdot C_X - fC_\alpha \qquad (4)$$

In the healthy organism all values, found in the right half of both equations, are constant, consequently the derivatives are equal to zero. From the equation it also follows, that change in the concentration of α-globulin occurs in three cases: varying the rate of the biosynthesis of X, varying the concentration of albumin or varying the rate of removal of α-globulin. Change in the concentration of albumin is the most interesting, since it establishes a relationship between both proteins. Equations 3 and 4 were verified by us on the basis of data of Rodionov and coworkers [18] and Uspenskaya [19].

These authors removed 50% of the blood in dogs, which was substituted with Ringer-Locke solution, and studied the process of plasma protein regeneration (determined the amount of total protein and its fractions). The authors showed that regeneration of the albumin described a smooth curve (Fig. 6, curve 1). The regeneration of α and β-globulins proceeds somewhat more quickly and gives curves with a maximum (for example, for α-globulin the characteristic curve III). In order to compare the data of the above-mentioned authors with equations 3 and 4 and determine the form of the function $C_A=f(t)$ we assumed that the regeneration of albumin is described by the equation:

$$C_A = \frac{C_1 + C_\infty \varphi_2 t}{1 + \varphi_2 t}, \qquad (5)$$

where C_∞ and φ_2 are constants, C_1 is the content of protein in zero time after bleeding, t is the time after bleeding. The constant φ_2, possibly, is the constant for the rate of biosynthesis of albumin. If we assumed that blood albumin arises from tissue resources, to this constant can be ascribed another physiological significance. Constant C_∞ is calculated close to the normal level of blood albumin.

We do not impart physical-chemical significance to all constants which figure in the considerations. It must be considered that they reflect a definite line of behavior of physiological regulatory mechanisms. Calculations made according to formula 5 are presented in Figure 6; these correspond with our experimental data. In order to determine the form of the curve for α-globulin with changing concentrations of albumin, it is necessary to solve

equations 3 and 4. simultaneously. However, the solution of this quadratic equation with a positive value of $C\infty$ in elementary functions is impossible, so that it must be solved numerically.* In order to solve the equation we took the following values of the constants: $\varphi_2 = 0.18$; $\varphi_1 = 1$; $C\infty = 3.35$; $f = 0.9$. As seen from Figure 6, the calculated curve (II) has almost all the properties of the experimental curve. The concentration of α-globulin increases more quickly than the concentration of albumin, and, in addition, it passes through a maximum, although the latter is not pronounced. If it is assumed that the deficit of albumin is not the sole reason for the accumulation of X in the tissues, then the maximum on the curve can be made more pronounced. In calculating the curve it was assumed that the transport ability of albumin begins to be restored at the same time and proportionally to its blood concentration. Actually derangement in the transport must still exist because of destruction of the hemodynamics. Consequently, the transport effectiveness of albumin will be less than its concentration. In order for the results of the calculations to coincide well with the experimental data, it was assumed that excess X, accumulating in tissues, exceeds by four times the X which is created as a result of the decrease in the albumin concentration. In addition, all points of the curve are multiplied by an empirical factor of 1.5. In this case the shape of the calculated curve corresponds well with the experimental curve (Fig. 6, Curve III).

This account affirms that experiments on blood regeneration, as in the sections presented previously, furnish evidence in favor of the existence of α-globulin as a product of transformation of albumin.

Discussion of data in the literature, obtained by isotope methods and immunoelectrophoresis. Maurer and Miller [1] injected albumin labelled with S^{35} into rats. Blood was drawn from the rats on the fourth and twenty-fifth day after the injection of the labelled protein. Plasma proteins were fractionated by means of electrophoresis on paper; S^{35} was found in all plasma proteins. According to the authors' calculations, the content of labelled amino acids in globulins was somewhat higher than that which would be expected if the labelled amino acids were in equilibrium with the total amino acids pool in the organism. On this basis the conclusion was reached that albumin might be transformed directly into globulin. The fact that the label was transferred from albumin to globulin was not subject to doubt and was verified many times. Furthermore Jeffay and Winzler [20] have found that it is possible to reverse the transfer of the label from β-globulin to albumin and other proteins. The views of Maurer and Miller were subjected to criticism by Campbell and Stone [21]. These authors in their own experiments used albumin labelled with two amino acids: C^{14}-alanine and C^{14}-lysine. The labelled protein was injected into rats; the animals were sacrificed and the ratio of the radioactive alanine and lysine was determined. It was established that in albumin this ratio is 0.5 and in β-globulin, 7.0; in γ-globulin, 9.0. According to these authors [21] the data obtained did not permit the conclusion that albumin was directly transformed into β- and γ-globulins. However, Campbell and Stone did not carry out investigations on α-globulin. Furthermore, their experiments provided definite evidence in favor of the possibility of transformation of γ-globulin into β-globulin, since the ratios of radioactivity in amino acids in both proteins were similar. We believe that the Maurer and Miller's idea regarding direct transformation of albumin into such specific proteins as γ-globulin, is not very probable. On the other hand, the feasibility of the transfer of albumin into α-globulin, or γ-globulin into β-globulin corresponds with experimental data presented in the present paper, and does not contradict the data of Campbell and Stone.

Evidence in favor of such a transformation is provided by immunoelectrophoresic data. It is known that on immunoelectrophoretograms the precipitation curves reach from the albumin side to the α-globulin, and from the γ-globulin side to the β-globulin; between both proteins discontinuities exist. Thus position of the curve, responds to the scheme of electrophoretic homogenization. The author of the method, Grabar [22], assumes that part of the β-globulins are according to their derivation γ-globulins (transformation, described in this article). Kaplanskii and coworkers [23] have shown that α-globulin is immunologically close to albumin. They assume that there are no significant differences between albumin and α-globulin.

Such a statement corresponds to our assertion made earlier [2] and in the present article, however, it obviously refers to only part of the proteins of this group.

It can be assumed that processes of transformation of the type described in this communication, take place not only in the blood protein system but also in other processes of protein complex formation in tissues.

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*Analysis of the equation was carried out at our request with the assistance of the Department of Mathematics of the V. G. Yas'kov Krimean Pedagogical Institute.

Especially, a similar change can be related to quinoid metabolism, since it was shown earlier by us [3], that quinones can cause a process similar to electrophoretic homogenization, it is thus related to the process of "stress," collagenization, etc.

SUMMARY

It has been established that electrophoretic changes in serum proteins under the influence of various chemical reagents are extremely monotypical and are expressed in an increase in the content of fractions moving with the rate of α_2- or β_1-globulin. Isolated albumin and γ-globulin of bull serum were changed in a similar manner as corresponding fractions of whole serum, but the degree of these changes was different. According to data from salt fractionation, the chemical reagents can increase the content either of euglobulins or pseudoglobulins.

The feasibility of transformation of albumin into α-globulin was also shown in experiments on isolated heart and kidney.

The data from the literature obtained by isotope methods, immunoelectrophoresis, and also experiments on regeneration of blood proteins were reviewed. It was shown that results found here corresponded with hypotheses stated earlier by us on the transformation of protein. It may be assumed that the mechanism of such transformation is related to the destruction of specific native structures of protein in the process of complex formation and the formation of proteins having structures resembling statistical coils.

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FRUCTOSANS FROM WHEAT GRAIN

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Many authors have indicated the presence of fructose compounds in the grains of rye and wheat [1-4]. The structure of these fructosans varies, apparently, both according to the degree of polymerization and according to the type of bond between the separate molecules of fructose. The biosynthesis of similar compounds in plants and their role in the process of metabolism has not been studied sufficiently.

Investigations in later years have shown that transport enzymes are very important in the conversions of polyfructosides. The enzyme invertase in particular, as many authors have shown, possesses not only hydrolytic but transferring activity also [5-9]. In mixtures containing sucrose and extract of barley leaves, Pavlinova and Kursanov [10] succeeded in demonstrating the synthesis of low molecular weight oligosaccharides brought about by the transferring activity of invertase on sucrose.

In wheat the fructose compounds are also formed in the leaves and depending on their transfer into other organs (stem, mature ear) experience more polymerization, apparently, under the influence of still other enzymes.

The existence of fructose compounds during starch accumulation has been known for a long time, but in wheat no tests have been carried out to determine the relationship of the relative weight in the total mass of reserve carbohydrates of these fructosans and their changes to the extent of grain ripening. The present paper is dedicated to a study of these problems.

As will be evident from the experimental part of this communication, we succeeded in showing that a sufficient quantity of fructosans of various molecular weights are present in the ripening grain. In addition to the high molecular weight fructosans, which are not split chromatographically, and the lowest molecular weight fructoside-sucrose, other fructosans, were found and identified in wheat grain which occupy an intermediate position between these limiting members of the series. It was also discovered that the relative content of each of the identified components of the fructoside complex of the grain varied according to the maturity of the grain, according to the accumulation of starch.

TABLE 1. Content of Carbohydrates in Maturing Wheat Grain. Nigriaristatum 3 variety, in % of dry weight of the grain

Date the sample was taken	phase of development of the grain	glucose	fructose	sucrose	oligosaccharides			water-soluble glucose-fructosans	starch
					1	2	slow-moving		
14 June	beginning of milk-ripeness	0.5	0.7	2.7	3.1	1.4	10.3	5.5	29.1
20 June	milk-ripeness	0.3	0.5	1.6	1.2	2.1	2.9	1.9	35.9
30 June	wax-ripeness	0.3	0.4	0.9	0.9	0.7	0.6	1.9	51.5
14 July	complete ripeness	0.0	0.0	0.6	0.6	0.4	0.4	1.6	60.5

TABLE 2. Relative Content of Glucose in Oligosaccharides of Grain

Number of oligosaccharide	Ratio of glucose: fructose	
	experiment 1	experiment 2
Sucrose	1:0,9	1:1,13
1	1:1,8	1:2,04
2	1:3,02	1:2,76
3	1:4,14	1:3,58
4—6	1:5,22	1:5,43

METHODS

Grains of two winter wheat varieties were used for the experiments: Nigriaristatum 3 and Erythrospermum 72. Samples were taken in various phases of maturity of the grain. Fixation and extraction were carried out in boiling 96% ethyl alcohol, after which the grain was extracted twice with 82% ethanol. The residual plant material was extracted with water at room temperature for 16 hours. The water soluble carbohydrates of the grain (glucofructosans were determined in the water extract after three-hour hydrolysis with 2% HCl. The determination of sugars in this case was carried out according to Bertrand's method.

The starch content in the grain was determined by the diastatic method. Alcohol extracts, containing a mixture of carbohydrates were combined, evaporated in a vacuum, treated with $Pb(CH_3COO)_2$ and Na_2SO_4 and diluted to a definite volume. The purified solutions were used for quantitative chromatographic separation on paper. Chromatography was carried out on slow filtration chromatographic paper from Voldarskii Leningrad Factory. A mixture of n-butanol-acetic acid-water (4:1:5) served as a solvent. In order to develop the sugars, urea (on the ketoses) and aniline phthalate (on the aldoses) was used.

Quantitative determination of the individual sugars and oligosaccharides after the chromatographic separation was carried out by an elution method, described by Pavlinova [11], using a micromethod for the determination of glucose according to Hagedorn-Jensen [12].

RESULTS OF THE INVESTIGATION

The results of experiments which characterize changes in the carbohydrate content in the maturing grain of wheat are presented in Table 1.

Aside from glucose, fructose and sucrose were found in the wheat grain by chromatographic separation in addition to another group of oligosaccharides even more highly polymerized. Data are given in Table 1 on the content of the simplest oligosaccharides most resembling sucrose, oligosaccharide 1; the more complex oligosaccharide 2, still moving easily and a group of polymerized compounds which do not proceed far from the starting point in chromatographic separation. In addition, carbohydrates insoluble in alcohol were extracted from the plant material with water and determined by the supplementary method of Bertrand (Table 1) after hydrolysis with 2% HCl. This carbohydrate fraction remained at the point of application on separation in a mixture of N-butanol-acetic acid-water (4:1:5); this was evidence of the higher polymerization of the water-soluble compounds.

It is evident from the data that quite remarkable changes in carbohydrate composition occur in the ripening grain during starch accumulation which are expressed in a decrease of all forms of carbohydrate (primarily the higher molecular weight carbohydrates).

The other variety of winter wheat (Erythrospermum 72) had the same qualitative composition of sugars and oligosaccharides in the grain as Nigriaristatum 3, and was characterized by the same qualitative direction alteration of carbohydrates content during grain ripening.

The next phase of our work was an investigation of the nature of the grain oligosaccharides.

In an experiment with Erythrospermum 72 grain a mixture of oligosaccharides soluble in 82% ethanol was chromatographically separated into three individual compounds (Figure 1). According to the diameter of the molecule, these substances were located on the chromatograms between sucrose and the starting line. The simplest compounds (1-3) advanced to a position behind sucrose, and the most complex remained close to the starting line (4-6). The oligosaccharides chromatographically separated from the mixture were eluted and the solution concentrated. The concentrated extracts were hydrolyzed with 0.25 N H_2SO_4 [13], purified of SO_4^{--} ions by barium carbonate. The $BaSO_4$ precipitate was separated from the solution by centrifugation. The sucrose solution purified in this way were again applied to the paper for chromatography. After this separation the sugar was eluted from the paper and quantitatively determined according to the Hagedorn-Jensen method.

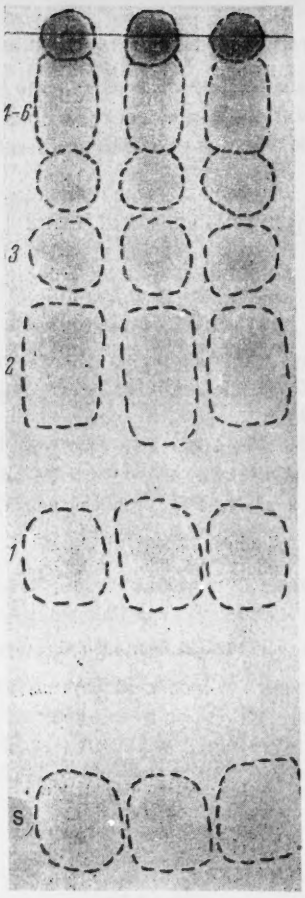

Fig. 1. Chromatograms of sugars and oligosaccharides of winter wheat grain, Erythrospermum 72 variety. S, sucrose; 1-6, oligosaccharides in order of increasing polymerization.

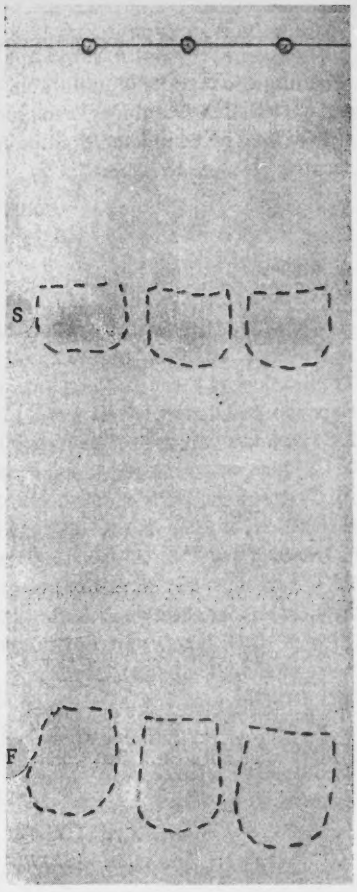

Fig. 2. Chromatograms of oligosaccharide hydrolyzates of winter wheat grain, Erythrospermum 72 variety. Hydrolysis with 0.25 N H_2SO_4; 70°, 10 min.; F, fructose; S, sucrose

Analysis of the partial (incomplete) hydrolyzates of grain oligosaccharides showed that the products of incomplete hydrolysis of these substances are sucrose and fructose (Figure 2). Analysis of the complete hydrolyzates showed that the oligosaccharides of grain are composed of glucose and fructose. Data are presented in Table 2 which characterize the relationship of glucose and fructose in the investigated oligosaccharides.

The data of Table 2 indicate (taking into account the possible errors of the method), that grain oligosaccharides in a series from sucrose to the highest polymer differ only by a single fructose residue. These data permit us to consider that they all are obtained from sucrose by means of the gradual annexation of one fructose residue.

It was found by analysis of the complete hydrolyzates of a mixture of water-soluble carbohydrates that these substances contain, in addition to fructose, a certain amount of glucose. As was shown above, these water-soluble glucofructosans remain at the point of application on chromatographic separation, this is evidence for their high polymerization; apparently, these sugars consist of not less than 6 hexose residues. Probably, low molecular weight oligosaccharides in the grain are formed by the transforming action of invertase on sucrose, and more highly polymerized fructosans are formed by other enzymes [14, 15].

SUMMARY

Oligosaccharides of wheat grain were separated by means of paper chromatography.

The oligosaccharides of grain were decomposed into glucose and fructose when completely hydrolyzed. In a series of isolated fructosides, each successive fructoside differed from the previous one by one fructose residue. It may then be considered that the formation of fructose compounds of grain occurs as a result of the transfer of fructose residues to sucrose.

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INCORPORATION OF LABELLED AMINO ACIDS INTO PROTEIN FRACTIONS OF THE CELL NUCLEI OF LIVER AND EHRLICH ASCITIC CARCINOMA

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The mechanism of biosynthesis of proteins has been studied less in cell nuclei than in cytoplasm. Only recently an investigation has appeared [1] indicating some parts of this process in nuclei and cytoplasm are similar.

The difficulty of obtaining and interpreting data on cell nuclei is related in large measure to the complexity of obtaining pure native nuclei from various tissues. In the majority of existing studies the total biosynthesis of protein was investigated in nuclei without attempting to fractionate the nuclear components. In a few investigations, in which fractionation of the nuclear components was carried out, cytological controls were absent, because of which it was difficult to follow the participation and relative activity of different nuclear structures in the biosynthetic processes.

In 1948, Zbarskii and Debov [2] developed a method of fractionating isolated nuclei into nuclear protein fractions, as well as acid fractions and residual proteins. Using this method, Zbarskii and Perevoshchikova [3, 4] studied the incorporation of labelled amino acids into proteins of nuclear fractions of normal organs and experimental tumors in vivo. The results demonstrated the relatively small metabolic activity of the tumor cell nucleus and some distribution of specific activity between the nuclear fractions. While in normal nuclei the most active fraction was the acid protein, in tumor nuclei the most active was the nucleoprotein fraction.

Busch and co-workers[5,6], during investigation of the biosynthesis of protein in nuclei and cytoplasm of tumor tissue, separated histone from the nucleus. Fractionation of the nuclear proteins and comparison of the incorporation of radioactive amino acids into various fractions was accomplished in Mirsky's laboratory [7-9]. However, the fact that the physical chemical properties of the nuclear proteins were the basis of the fractionation, and that their intranuclear location remained for the most part obscure, limited the opportunity for systematic comparison of the biosynthesis of protein in various nuclear structures.

Cytological characteristics of the nuclear fractions were given by Zbarskii and Georgiev [10, 11], showing that fractions successively extracted with 0.14 M NaCl, 1.5 M NaCl and 0.02 M NaOH, were identical respectively, with material of the nuclear sap; desoxynucleoprotein (DNP) of chromatin, nucleolus and "residual chromosomes" and membrane material.

In as much as a number of papers [3-6, 12, 13] have shown that the biosynthesis of protein is disturbed in tumor cell nuclei, it was most interesting to compare the metabolic acitivity of the nuclear structures of tumor cells with those of normal cells. In the present paper an in vivo investigation is presented involving the incorporation of C^{14}-amino acids into protein of different cytologically characterized fractions, separated from the isolated cell nuclei of liver and Ehrlich ascites carcinoma. At the same time, the weight relationship of these protein fractions in the two types of tissue was investigated.

METHODS

Nuclei from the liver of normal rats and cells of Erhlich asictes carcinoma formed the experimental material for this investigation. The experimental animals were injected with radioactive amino acids (glycine-C^{14}, tyrosine-C^{14} or lysine-C^{14}). After varying periods of time (6-90 min) the animals were killed, and the nuclei were separated from the liver, previously perfused for the removal of blood with 0.14 M NaCl and 0.25 M sucrose, according to the method of Chauveau [14], modified by Georgiev and others [15].

In order to separate the nucleous from cells of Ehrlich ascites carcinoma, collected by puncture of the peritoneal cavity 7-8 after transplantation, we ruptured the cellular membrane by means of osmotic shock [16].

To cells of ascitic carcinoma, separated by centrifugation from the ascitic liquid and washed three times with 0.075 M K_2HPO_4 (pH 8.0), 12-15 times their volume of distilled water was added which caused osmotic rupture of the cellular membranes. After 15-17 min the nuclei were separated from the cytoplasm by centrifugation (5 min at 700 g). The nuclear precipitate was washed out from the cytoplasm 3-4 times (to the disappearance of turbidity in the supernatant liquid) with 0.25 M sucrose, to which 0.15% K glycerophosphate was added in order to prevent excessive swelling of the nuclei. After each washing the suspension of nuclei was centrifuged at 700 g for 5 min.

The osmotic shock method made it possible to obtain nuclei contaminated only slightly with cytoplasm; however, it can be assumed that some loss and destruction of the globulin fractions resulted from the use of this method; this is the possible explanation for small amount of this fraction extracted from nuclei of the ascitic cells, and their low content of RNA. The purity of the separated nuclear preparation was verified by a microscopic smear of the nuclei after staining with pyronine-methyl green. In most of the preparations we rarely found cytoplasmic impurities; occasionally they were caught in the connective tissue fibers.

These nuclei were subjected to successive extraction with 0.14 M NaCl, 1.5 M NaCl, and 0.05 M NaOH resulting in a globulin fraction appropriate to material from the nuclear sap; a DNP fraction; an acid protein fraction, representing the RNA and protein of the nucleolus and "residual chromasomes" [10]; and the residual protein. The latter was first discovered by Zbarskii and Debov [2, 17, 18] and is, in accordance with data of Soudek and Benes [19] nuclear membrane material.

In a series of experiments separate histone and nonhistone protein were separated from the DPN fraction. To accomplish the separation $^1/_4$ volume of 2N H_2SO_4 was added to the DPN solution in 1M NaCl. The precipitating residue of nonhistone protein together with DNA was separated by centrifugation from the histone solution, which was precipitated with TCA (final concentration 13%). All procedures in separating and fractionating the nuclei were carried out in the cold room at a T of 2-4°. The proteins of the extracted fractions were precipitated with TCA (final concentration 7-10%) in calibrated centrifuge tubes, washed 2-3 times with 5% TCA and then were freed of fat and dried with alcohol, a mixture of alcohol and ether, and ether. The dry preparations thus obtained were weighed in order to determine the weight of each fraction.

The radioactivity of the preparations was determined by means of an end-window counter in small weighed portions of protein (1-2 mg), applied in a uniform layer to circular metal planchettes. The specific activity obtained was the initial value for the calculation of the relative specific radioactivity, consisting of the ratio of the radioactivity (counts/min) contained in 1 g of dry preparation, to the radioactivity injected per 1 g body weight of the animal.

RESULTS AND DISCUSSION

The proteins content in various fractions of the cellular nuclei of rat liver and Ehrlich ascitic mouse carcinoma were shown in the following (in % dry weight; average values from 8 experiments):

Investigated nuclei	Globulin fraction	DNP	Acid protein	Residual protein
Liver	25.9(±1.3)	64.3(±1.4)	4.6(±0.6)	5.4(±1.2)
Ascitic carcinoma	17.8(±1.17)	65.2(±2.1)	9.1(±0.4)	7.6(±1.5)

As seen from these data, the liver cell nuclei and Ehrlich ascitic carcinoma cell nuclei differed somewhat from each other in protein content in the globulin fraction and the acid protein fraction. In nuclei of ascitic

cell nuclei the globulin fraction was less, but the acid fraction more, than in nuclei of liver cells. The smaller amount of globulin fraction in ascitic cell nuclei can be partially explained by the nature of the method of separating the nuclei from the ascitic cells, by which part of the globulin fraction can be lost. More acid protein in ascitic cells than liver nuclei, possibly, is characteristic of the tumor cells, and apparently reflects a greater development of the nucleolar apparatus, in the composition of which this protein is involved [15]. On the other hand, in previous work [2, 12, 17, 20] more acid protein was found in the nuclei of normal tissues, as well as in malignant and benign tumors and regenerating liver. Part of these differences can be explained by the fact that in the above mentioned studies the globulin fraction was not considered, so that the percentage of other fractions was correspondingly higher. Other reasons for this discrepancy can be that the nuclei were less free of cytoplasmic contamination and the method of separation of the nuclei was different.

The total amount of protein, joined to DNA in the ascitic cell nuclei approximates the corresponding value for liver nuclei. In ascitic nuclei a larger amount of residual protein in comparison with liver nuclei was also noted. This observation corresponds to results of previous work [2, 17, 20-22]. In accordance with the studies mentioned, the residual protein in tumor nuclei was, however, considerably higher. The reason for the discrepancy in this respect remains obscure, although part of it can be explained by other methods of studying the tumors and differences in the method for separating the nuclei.

In order to characterize the changes in radioactivity in various protein fractions of the liver nucleus and ascitic cells, we investigated the incorporation of radioactive amino acids at various times after their injection. We were interested in beginning the investigation with short periods of exposure to radioactive amino acids, in order to discover, if possible fraction in the nucleus, which would incorporate the labelled amino acid most quickly. Similar experiments on the incorporation of labelled glycine-C^{14} and tyrosine-C^{14} in nuclear protein of rat liver are presented in Table 1.

As seen in Table 1, the incorporation of glycine-C^{14} and tyrosine-C^{14} are similar. In some experiments glycine-C^{14} was incorporated at a higher rate than tyrosine-C^{14}. During short intervals of time (5 min) the incorporation of glycine-C^{14} in whole liver homogenate was higher than in any nuclear fractions. This, probably is explained by the fact that the labelled amino acid in such a short time does not succeed in penetrating the nuclear membrane. However, even at 15 min. after injection of the radioactive amino acids some fractions of the nucleus (acid protein) as measured by their radioactivity approach the homogenate.

In general, the acid protein of the liver nuclei during short-term exposures is the most radioactive protein. At 5-15 min after injection of the labelled amino acid its radioactivity is demonstrated to be 50-88% higher than the radioactivity of the globulin protein fraction, which also possessed high radioactivity. At 30-60 min the radioactivity of the acid protein fraction approaches the radioactivity of the globulin fraction and the non-histone protein. The incorporation of amino acids in the non-histone protein was also significant and after 30-60 min exposure in a series of experiments even exceeded the incorporation in the globulin fraction. Histones of

TABLE 1. Incorporation of Glycine-C^{14} and Tyrosine-C^{14} in Nuclear Proteins of Rat Liver. Ratio of counts/min/g of protein to counts/min/g body weight

Time after injection, min	Radioactive amino acid	Relative specific activity of fraction						
		globulin	DNP	histone	non-histone protein	acid protein	residual protein	homogenized whole tissue
5	Glycine-C^{14}	0.47	0.25			0.89	0.083	2.8
15	"	2.46	1.29			3.98	0.42	4.05
15	"	3.62	2.22			5.15	0.91	6.05
15	Tyrosine-C^{14}	0.57	0.81	0.47	1.39	2.38	3.65	3.04
30	Glycine-C^{14}	4.47	3.15			5.82	0.081	6.4
30	"	7.15	4.85			8.80	0.58	9.2
30	Tyrosine-C^{14}	3.28	2.62	0.87	3.38	3.93	1.12	5.25
30	"	3.38	3.05	0.83	5.04	4.77	1.73	4.85
60	Glycine-C^{14}	7.28	4.72			5.16	1.44	8.04
60	"	8.75	6.00			7.62	0.98	
60	"	9.00	5.20			8.85		
60	Tyrosine-C^{14}	4.01		1.41	5.16	5.05	2.23	5.12

the liver nuclei had little radioactivity, 3-6 times less than the non-histone protein. No regularity was found between rate of radioactive uptake in relation to the non-histone protein.

The incorporation of glycine-C^{14} in the residual protein was 5-10 times lower, than in the globulin fraction; the radioactive tyrosine was incorporated at a somewhat higher rate than glycine. Thus, injected amino acid was the most rapidly incorporated in proteins localized in nucleolus material and residual chromatin [10, 11, 24]. Radioactivity of the proteins of the globulin fractions, representing the nuclear sap fraction, was also very large, although during short exposures it was somewhat less than the radioactivity of the acid protein.

The radioactivity of proteins bound to DNA in the liver nuclei, varied widely in relation to the form of protein. The radioactivity of the non-histone protein approached that of the proteins of the globulin fraction and acid proteins, the most intensively incorporated label. The histone incorporated tyrosine-C^{14} and glycine-C^{14} is 3-6 times less intensive than the non-histone proteins. The radioactive amino acids incorporated in the residual proteins representing the proteins of the nuclear membrane had the lowest rate, although tyrosine-C^{14} was incorporated in these proteins more intensively than glycine-C^{14}.

Distribution of radioactivity in protein fractions of ascitic cell nuclei is presented in Table 2. The incorporation of the three amino acids into the nuclear proteins varied. The most actively incorporated was tyrosine C-^{14}, the incorporation of glycine-C^{14} was somewhat less, and lysine-C^{14} incorporation was almost 10 times lower than tyrosine uptake. The proteins differed considerably with respect to change in radioactivity of the various fractions with time, thus the lysine-C^{14} incorporation differed from glycine-C^{14} and tyrosine-C^{14} in this regard. The two latter amino acids were taken up similarly, differing only by a higher total level tyrosine incorporation .

In ascitic carcinoma nuclei, in contrast to liver nuclei, the incorporation in the globulin fraction was equal or even exceeded the incorporation in the acid protein during short intervals of time (15-30 min). During the longer time (60 min), on the contrary, tyrosine-C^{14} and glycine-C^{14} uptake in acid protein was higher than in the globulin fraction. This result corresponds with that obtained in the studies referred to of Zbarskii and Perevoshchikova [3, 4]; they noted that there was less glycine-C^{14} and tyrosine-C^{14} incorporation in rat sarcoma M-1 when compared to incorporation in the acid protein of rat liver nuclei.

The incorporation of the label in histones of ascitic cell nuclei somewhat surpassed the corporation in histones of the liver nucleus and was approximately 70% of the incorporation in non-histone protein. A tendency was noted towards an increase in the per cent incorporation in histones as far as increase in time of incubation with the labelled amino acid was concerned. Thus, the 15 min uptake in histones was 50-51% of the incorporation in non-histone protein in 15 min, 70-71% in 30 min, and at 60 min, incorporation in the histones became even higher and was only 14-15% lower than incorporation in non-histone proteins.

The incorporation of the label in acid protein during short intervals of time approximated the incorporation of the labelled amino acids in the non-histone protein, but was lower than in the globulin fraction. At 60 min the radioactivity of the acid protein became higher than the globulin fraction. The incorporation of the label in the residual protein was 3-5 times lower than incorporation in the most active fraction.

The incorporation of lysine-C^{14} in different fractions of the ascitic carcinoma nucleus in some respects is different from their uptake of glycine-C^{14} and tyrosine C-^{14}. Besides a decrease in the total rate of incorporation in all fractions, an increase was noted also in the level of incorporation of lysine-C^{14} in protein of the DNP complex, as a result of which the non-histone protein became the most radioactive fraction of the nuclear proteins, and incorporation in histones was only 5-8% less.

Busch and others [5, 6], also point out the high incorporation of lysine-C^{14} in histones of tumor tissue nuclei in which the radioactivity during short intervals after injection of labelled amino acids exceeded that in other nuclear and cytoplasmic fractions. Difference in the investigated tumors and in the methods of fractionation of the nuclear proteins in our work and in the work cited above does not make a more detailed comparison possible and can explain some differences from our data.

The globulin fraction in all investigated intervals of incubation possessed a lower radioactivity than non-histone protein and even histones. Attention is called to the lower radioactivity of the acid protein. The low incorporation of lysine-C^{14} in the acid protein is explained, possibly, by the small content of lysine in proteins of this fraction; similarly the high specific radioactivity of the histone on incorporation of lysine-C^{14} can be

explained not only by the relatively high rate of metabolism of this fraction in ascitic cells but its large content of lysine [25].

There are no references in the literature to amino acid composition of acid protein of ascitic cell nucleus. However, there are indications of the higher content of lysine in acid protein of malignant tumor nuclei in comparison with the normal [17].

Lysine-C^{14} is incorporated relatively vigorously in residual protein, the radioactivity of which was only 1.5-2.5 times less than the nuclear acid protein.

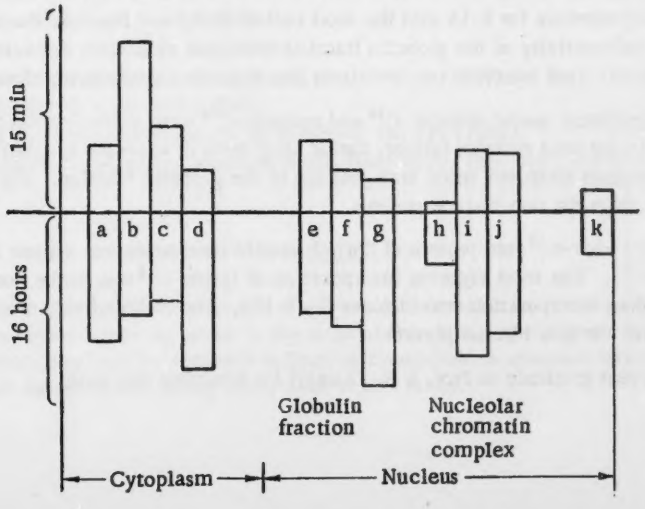
In order to be able to determine the distribution of radioactivity in various separated fractions and to reach a conclusion regarding the dynamics of these changes and the relative rate of metabolism of protein in one or another of them, it is necessary both to know and consider their amino acid composition, and to compare the incorporation of some amino acids in various fractions.

In our work we investigated the incorporation of three amino acids, two of which were qualitatively similar with respect to incorporation in various nuclear proteins; obviously, they can indicate through their uptake the relative rate of metabolism of each of these fractions. Lysine-C^{14} incorporation possibly is characterized by the fluctuation of its content in various fractions; a relatively small amount in acid protein and large amount in histone (accompanied by an increase in the rate of metabolism of the latter). Our data, in accordance with the data of Zbarskii and Perevoshchikova, on the significant and rapid incorporation of radioactive amino acids in normal liver acid protein localized, as it was already stated, in the nucleolus and residual chromatin, and also some data in the literature which shows the role of the nucleolus in the biosynthesis of protein and RNA, are evidence that the synthesis of some proteins, and possibly, of ribonucleoprotein granules, can occur in the nucleolar apparatus.

Investigating the changes with time of radioactive amino acids uptake in different nuclear fractions, it was interesting also to compare the radioactivity in nuclear fractions with the radioactivity in cytoplasmic fractions. Data on the relative radioactivity in nuclear and cytoplasmic fractions are compared in the diagram.

Among the other fractions in the diagram are presented the radioactivity of three fractions from which nuclear sap material can be separated by means of ultracentrifugation [26]. Two of these fractions are granular and according to several criteria (precipitation with ultracentrifugation, RNA content, etc) are similar to microsomes and ultramicrosomes of cytoplasm; the third is a soluble fraction of the nuclear sap. We gave a more detailed

Diagram of relative specific activity of various fractions of the cytoplasm and nucleus of rat liver at 15 min and 16 hours after injection of glycine-C^{14}. Key: a) mitochondria; b) microsomes; c) ultramicrosomes; d) soluble fraction; e) nuclear microsomes; f) nuclear ultramicrosomes; g) supernatant fraction of nucleus; h) histones; i) non-histone protein; j) acid protein; k) residual protein.



description of these fractions in the work referred to [26]. As seen from the diagram, after 15 min radioactivity of the nuclear fractions, among which the highest label was in the nuclear "microsomes" [26] of the globulin fraction as well as in the non-histone and acid proteins of the DNP and nucleolar-chromatin complex. In cytoplasm the highest radioactivity was found in the microsome fraction, which corresponds well with data in the literature. At 16 hours exposure to the labelled amino acid the radioactivity of the majority of nuclear proteins became higher than the radioactivity of the cytoplasmic proteins. The large and almost equivalent radioactivity of the supernatant fraction both in the nuclear sap and in the cytoplasm was especially characteristic. On the contrary, the radioactivity in the microsome fractions of the nucleous and the cytoplasm increase only slightly and was somewhat less than that in the soluble fraction. The relationship of the radioactivity in various fractions of the nuclear sap at 15 min exposure to the labelled amino acid contrasted with this relationship after 16 hours exposure.

The radioactivity of all fractions of nucleolar-chromatin complex (histone, non-histone protein and acid protein in the nucleus residual protein also markedly increased while the relationship between the irradioactivities did not change.

Thus, 15 min after injection with the labelled amino acid a considerable amount of radioactivity could be be found in some rat liver nuclear fractions in comparison with radioactivity of some fractions of cytoplasm. If it is considered that during short time intervals the concentration of radioactivity of the nuclear amino acids is probably lower than in the cytoplasm, we must assume that synthesis, at least of some proteins of the nucleus, proceed rapidly, not falling short of, but possibly even exceeding, the rate of biosynthesis of protein in the cytoplasm.

The rapid appearance of radioactivity and the high label of proteins in the globulin fraction, and also the presence of the ribonucleoprotein moiety in this fraction [26] can be an indication of the basic, while still not interpreted role of this fraction in the biosynthesis of nuclear protein. It can be assumed, that radioactivity of this fraction is not only the result of the passage into it of protein synthesized in other nuclei, and possibly in cytoplasmic fractions, but depends on synthesis of soluble proteins in the ribonucleoprotein granules themselves These proteins are later transferred and accumulated in the soluble part of the nuclear sap, causing an increase with time in its radioactivity.

The intensive accumulation of amino acids in histones of ascitic cell nuclei, that is, cells having a high mitotic activity, is possibly, a peculiarity of these cells which is related to protein biosynthesis of the desoxyribonucleoprotein complex in the process of its replication during mitosis.

SUMMARY

The weight composition and the incorporation in vivo of glycine-C^{14}, tyrosine-C^{14} and lysine-C^{14} in proteins localized in various structures of the nucleus of Ehrlich ascitic mouse carcinoma and normal rat liver were investigated.

Nuclei of Ehrlich carcinoma ascitic cells contain more acid protein, corresponding to material in the nucleolus and residual chromatin, and somewhat more residual protein, than liver cell nuclei.

In liver nuclei during exposure for 5-15 min the most radioactivity was found in the acid protein; on exposure for 30-60 min the radioactivity of the globulin fraction increased more than the acid protein fraction. The radioactivity of histones in all time intervals was 3-6 times less than the radioactivity of the non-histone protein.

In Ehrlich ascitic carcinoma nuclei glycine-C^{14} and tyrosine-C^{14} were actively incorporated in proteins of the globulin fraction and in the acid protein; further, during long-term of exposure to labelled amino acids, the radioactivity of the acid protein increased more than proteins of the globulin fraction. The radioactivity of the histone was 30-35% lower, than the non-histone protein.

The incorporation of lysine-C^{14} into protein of Ehrlich ascitic carcinoma was almost 10 times less, than glycine-C^{14} and tyrosine-C^{14}. The most vigorous incorporation of lysine-C^{14} was in the non-histone protein, which was somewhat less than incorporation into histones (by 5-8%). The radioactivity of the acid protein was almost two times lower than the non-histone protein.

I wish to express deepest gratitude to Prof. I. B. Zbarskii for directing this work.

TABLE 2. Incorporation of Glycine-C^{14}, Tyrosine-C^{14} and Lysine-C^{14} in Various Proteins of the Nucleus of Ehrlich Ascitic Carcinoma. Ratio of counts min/g protein to counts/ min/g body weight

Time after injection, min	Radioactive amino acid	Relative specific activity of the fraction					
		globulin	DPN	histone	non-histone protein	acid protein	residual protein
15	Glycine-C^{14}			1.06	1.52	1.27	0.20
15	"	1.85		1.00	1.92	1.47	0.4
15	Tyrosine-C^{14}	4.15		2.55	3.88	3.96	0.61
15	Lysine-C^{14}	0.42		0.52	0.57	0.32	0.25
30	Glycine-C^{14}	6.25	4.61	3.58	5.06	5.68	2.24
30	Tyrosine-C^{14}	8.20	6.17	6.32	8.74	10.82	4.03
30	Lysine-C^{14}	1.00		1.40	1.50	0.82	0.40
60	Glycine-C^{14}			1.78	22.5	22.3	
60	"	16.32		14.00	14.00	24.00	6.65
60	Tyrosine-C^{14}	29.00		28.00	32.16	37.33	3.75
60	Lysine-C^{14}	2.00		1.97	2.12	1.25	0.48

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GLYCOGEN IN THE ORGAN OF CORTI UNDER RESTING CONDITIONS AND DURING ACOUSTIC STIMULATION

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Vinnikov and Titova [1] worked out a method for isolating the surviving labyrinth and thereby made it possible to subject the organ of Corti, isolated while still alive, to detailed histochemical study. In addition to other substances contained in the organ of Corti, they investigated the presence and distribution of glycogen and established that glycogen was concentrated in the external hair cells and was found under resting conditions in small transparent granules, which filled the whole cytoplasm more or less evely, except for the nucleus [2]. In some hair cells there were very few glycogen granules, and the granules could even be completely absent. During an hour's acoustic stimulation the properties of intracellular glycogen altered markedly. It lost its granular appearance and was diffusely distributed throughout the whole cell. The number of cells in which glycogen was absent increased noticeably.

The histochemical method used by Vinnikov and Titova gave only a very approximate indication of quantitative changes in glycogen during acoustic stimulation. To obtain a more exact picture of the quantity of glycogen in the organ of Corti and of the changes produced by acoustic stimulation requires the method of quantitative biochemical analysis. This aim has been realized in the present study.

Besides determining the total quantity of glycogen in the organ of Corti, we also investigated its separate fractions. As noted by many authors, intracellular glycogen is not homogeneous: part of it is easily extracted, while part is extracted only with difficulty.

METHODS

The experiments were carried out on guinea pigs. The cochlea was isolated according to the method described by Vinnikov and Titova [1]. The animals received no food for 16-18 hours preceding the experiment.

The experiments can be divided into two series. In one series the intact animals were subjected to acoustic stimulation. As soon as the sound was turned off, the animals were decapitated, and the cochlea isolated from the bone. In these experiments, animals which had not been subjected to acoustic stimulation served as controls. In the other series of experiments the cochlea was isolated at the beginning of the experiment. The cochlea from one side was immersed in Ringer's solution and placed in a thermostat at 37° with a sound source a dynamo combined with a sound generator. The sound was at 95 db and 1500 cps. Stimulation lasted for an hour. The cochlea from the other side served as the control and was immediately placed in a test tube for analysis. Under normal conditions the cochlea from both sides contain similar quantities of glycogen.

Glycogen was determined by the method of Kemp and Kits van Heijningen [3]. For determination of total glycogen the cochlea was frozen either with solid CO_2 or liquid nitrogen. In individual cases freezing was omitted, with no effect on the glycogen content. After weighing, the cochlea was dissolved in a solution of

80% methanol. The final determinations were carried out according to the method cited, except that the volume of TCA was 2.5 ml rather than 5 ml.

When glycogen fractions were determined, the first extraction was carried out at room temperature. The cochlea was dissolved in 2.5 ml of a 5% TCA solution containing 0.1% $AgSO_4$ to precipitate chlorides. The supernatant was subjected to analysis, while a fresh aliquot of acid was added to the precipitate, and extraction was continued at the boiling point. The fraction obtained by extraction at room temperature will henceforth be termed fraction I, and fraction II is the fraction obtained at the boiling point.

One must bear in mind that the first fraction contains, in addition to glycogen, other substances, of which glucose and glucose-1-phosphate give a color reaction in the remainder of the procedure. Glucose-1-phosphate is generally present in very small quantities. In our experiments, as we determined, it could be neglected. A glucose determination was carried out on the supernatant of the methanol solution remaining after determination of total glycogen [3]. By subtracting the glucose content from fraction I, we could estimate the free glycogen. To extract particularly firmly bound glycogen, the tissue residue was treated with hot 60% KOH, after which the solution was neutralized and glycogen was precipitated with 80% methanol. TCA (2.5 ml, 5%) was added to the precipitate; the solution was boiled, and glycogen determined in the supernatant as in the preceding fractions. This fraction will henceforth be termed fraction III.

Colorimetric determinations on solutions were carried out in a FÉK-M-1 photoelectric colorimeter. Although the quantity of glycogen in the samples was very small and the error of individual determinations rather large, it was still possible to do a determination with sufficient accuracy to detect the absence or presence of changes during acoustic stimulation.

To have a check on the results and to increase the glycogen content in the samples, we conducted further experiments in which the cochleae from one side of five or six animals were analyzed together. In one experiment the residual glycogen from five guinea pigs was determined by the method of Brand [5], one of the adaptations of Pfluhger's method on a micro scale. All results were analyzed statistically by the method of small samples [6].

RESULTS

The total glycogen content in both cochlea of a guinea pig under normal conditions varied approximately from 50 to 100 μg. The average value in our experiments was 69 μ g (standard error ± 6); i.e., each cochlea contained about 35 μg of glycogen. From the weight of the whole cochlea, the glycogen content on a percentage basis was about 0.5%. However, as established by Vinnikov and Titova, almost all the cochlear glycogen is concentrated in the organ of Corti. This findings were confirmed in the present study. Experiments showed that a cochlea from which the organ of Corti had been removed contained practically no glycogen, or only traces. From data in the literature [7] about the dimensions of the organ of Corti in guinea pigs, one may conclude that it weighs approximately 0.2-0.3 mg. Thus, the glycogen content of the organ of Corti can be taken as about 10-15%, i.e., this organ is very rich in glycogen.

Our experiments also showed that almost all the glycogen of the organ of corti is in the bound form. Fraction I consisted almost entirely of glucose (see table).

We did not succeed in detecting any differences in the glycogen content in the organ of Corti from guinea pigs under resting conditions and subjected to intense acoustic stimulation for one hour (Fig. 1). The insignificant decrease in glycogen content from animals subjected to acoustic stimulation did not exceed the limits of individual variation. This decrease could not be considered statistically significant ($p > 0.25$). Nor did we succeed in detecting any changes in the content of separate glycogen fractions (Fig. 2).

Different results were obtained in the other series of experiments. In these experiments acoustic stimulation was used not on the whole animal, but on the isolated cochlea. The results of this experiment are depicted in Fig. 3. The results show that when the isolated cochlea is subjected to acoustic stimulation, the content of total glycogen, glucose, and also of fraction I does not change. However, the quantity of glycogen in fraction II, unlike all the previously mentioned components, decreased sharply under the influence of sound. The decrease in content of bound glycogen was caused by sound but not by immersion in Ringer's solution for an hour at 37° (Fig. 4).

It is easy to see that all or nearly all of fraction I consist of glucose, i.e., all or nearly all the glycogen in the organ of Corti is in the bound form. Some scatter in the values and their ratios in different groups of experiments may probably be the explanation for the rather large individual variation; one must keep in mind that the experiments were carried out at various times of year on guinea pigs supplied in different batches and kept under different conditions. This probably also explains the circumstance that the quantity of bound glycogen exceeds the quantity of total glycogen (Figs. 3 and 4). Of course, one cannot exclude the possibility that some part of the total glycogen is lost during its determination, but this could not affect the results of the experiments.

Naturally, the question arises: how can it happen that the total quantity of glycogen does not change under the influence of sound, while the quantity of bound glycogen decreases without a corresponding increase in free glycogen? It might be supposed that the increase in free glycogen is undetectable because an equal quantity of glucose is used up by the organ; in this case the sum of free glycogen and glucose, constituting fraction I, should not change. However, this explanation is unacceptable, because the quantity of glucose remains unchanged during acoustic stimulation. The possibility remains that after insonation part of the glycogen forming fraction II is no longer extracted even by hot TCA, in other words, it becomes even more difficult to extract from the tissue, and even more firmly bound.

Experiments carried out to test this assumption confirmed it. The quantity of glycogen in fraction III increased under the influence of sound (Fig. 5). The same result was obtained in other experiments in which the total quantity of glycogen was determined in five cochlea from the same side, while in one of the experiments the residual glycogen (fraction III) was determined after alkaline hydrolysis of the tissue by precipitation with ethyl alcohol, followed by acid hydrolysis (according to the method of Brand).

Evidently the preliminary treatment of the tissue with methyl alcohol upset the normal binding of glycogen by the tissue. Otherwise it would be difficult to explain why sound has no effect on the total quantity of glycogen.

DISCUSSION OF RESULTS

The results given above show, first of all, that the organ of Corti is very rich in glycogen. This fact provides a basis for supposing that glycogen plays some important role in the reception of sound. In this connection it is worth remembering the experiments of Wever [8], Fernandez [9], and other authors showing that the organ of Corti and especially its receptors, the hair cells, have a high metabolic rate and are vey sensitive to lack of oxygen. It was also shown that the

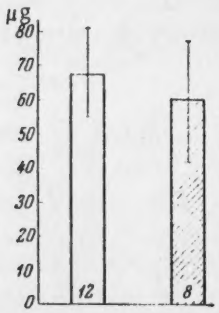

Fig. 1. Total glycogen content in the organ of Corti of guinea pigs, from experiments involving acoustic stimulation of intact animals. Blank columns give the average glycogen content in both organs of Corti from the control animals. Shaded columns refer to animals subjected to acoustic stimulation. The number at the bottom of each coumn is the number of animals used. Vertical lines are confidence limits for p=0.05.

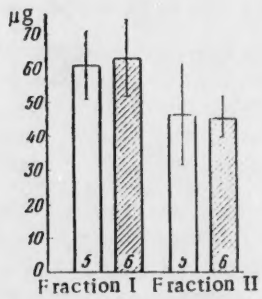

Fig. 2. Fractions I and II in the organ of Corti of guinea pigs, from experiments involving acoustic stimulation of intact animals. Symbols as in Fig. 1.

Breakdown of Carbohydrates of the Organ of Corti into Fractions

Carbohydrates	M, μg	S.E. M.
Total glycogen	31	2.5
Glucose	27	2.5
Fraction I	32	2.5
Fraction II	24	2.0

Note: Results obtained from analysis of the cochleae from 14 animals. Total glycogen and glucose were determined in the cochleae from one side, and fractions I and II were determined in the cochleae from the other side; M is the average content, in μg; S.E.M. is the standard error of the mean.

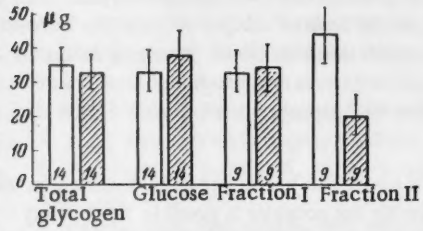

Fig. 3. Carbohydrate fractions in the organ of Corti of guinea pigs, from experiments involving acoustic stimulation of the isolated cochlea. Blank columns refer to control cochlea, shaded columns to cochlea subjected to acoustic stimulation. Symbols as in Fig. 1.

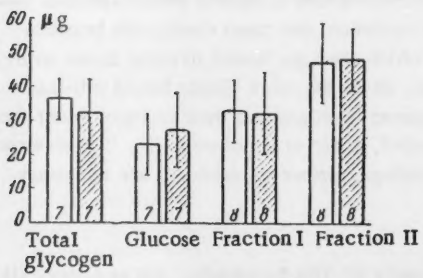

Fig. 4. Carbohydrate fractions in the organ of Corti of guinea pigs, from experiments in which one cochlea served as control, while the other was left in Ringer's solution for an hour at 37°. Symbols as in Fig. 1.

electrical activity of the cochlea appearing during acoustic stimulation is not fully abolished by anoxia, but can be partially maintained even for several hours after the death of the animal. This indicates that the organ of Corti is capable of significant anaerobic metabolism. One must suppose that under both aerobic and anaerobic conditions the principal energy reserve of the cochlea is carbohydrate. These reserves cannot be directly supplied by the blood to the organ of Corti, since it lacks blood vessels. As shown by Wing [10], a fall of blood sugar level to 40 mg%, or, according to the results of Fernandez [9], even to 20 mg%, does not lead to abolition of the cochlear potential. Evidently the rich reserves of glycogen contained in the organ of Corti are used under these circumstances.

Nevertheless, we were not able to show any decrease in glycogen content upon prolonged and intense insonation. Nor did we find any changes in the separate glycogen fractions in the experiments on intact animals. This result is not in accord with the evidence of Vinnikov and Titova, who observed changes in the histochemical pattern of glycogen during acoustic stimulation. Perhaps this discrepancy is explained by the rather significant individual variations in glycogen and its fractions among experimental guinea pigs, and against this background we could not have detected small changes even under conditions where they were occurring.

In the experiments on isolated cochlea we observed distinct changes in glycogen fractions upon insonation. Part of the bound glycogen became even more resistant to extraction from the tissue; it could not be extracted even with hot TCA, and to release it treatment of the tissue with concentrated alkali was necessary. During insonation the glycogen of the organ of Corti apparently becomes even more firmly bound to some tissue components, probably proteins. However, the interpretation of this fact is especially difficult, since the physiological role of the separate glycogen fractions is completely unclear.

Most authors follow Wilstatter and Rhodewald [11] and Przylecki [12] in classifying glycogen as free or bound, i.e., lyoglycogen or desmoglycogen. Numerous investigations have been carried out to clarify the functional significance of these fractions, but at present authors have not yet been able to come to any general agreement.

Russel and Bloom [13] studied glycogen fractions in liver, muscles, heart, and diaphragm under very diverse conditions and concluded that the content of bound glycogen was relatively constant; the fraction that was active in a physiological sense was lyoglycogen. Kits van Heijningen and Kemp [4] could not confirm the constancy of bound glycogen content in muscles but found that the decrease in glycogen during muscular activity proceeded chiefly at the expense of the free fraction. On the other hand, Kits van Heijningen [14] observed an increase in free glycogen in the diaphragm from the effect of insulin, and, proceeding from the fact that insulin favors the accumulation of glycogen reserves in muscles, supposed that the reserves are accumulated in the form of free glycogen. Meyer and co-

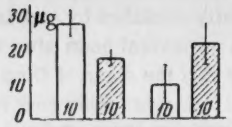

Fig. 5. Fractions II and III in the organ of Corti of guinea pigs, from experiments involving acoustic stimulation of the isolated cochlea. Symbols as in Fig. 1.

workers [15] also established that glycogen synthesis in heart muscle homogenate leads initially to the formation of free glycogen. However, Wajzer [16] concluded on the basis of his experiments that glycogen is synthesized as the bound fraction and consumed as the free fraction. Laureau and Meyer [17] used isotopic tracers and concluded that both fractions are synthesized independently. Kaikina and Goncharova [18] studied the rate of resynthesis of both fractions in brain under various physiological conditions and assumed that the free form of gly-

workers [15] also established that glycogen synthesis in heart muscle homogenate leads initially to the formation of free glycogen. However, Wajzer [16] concluded on the basis of his experiments that glycogen is synthesized as the bound fraction and consumed as the free fraction. Laureau and Meyer [17] used isotopic tracers and concluded that both fractions are synthesized independently. Khaikina and Goncharova [18] studied the rate of resynthesis of both fractions in brain under various physiological conditions and assumed that the free form of glycogen is the more active metabolically. Stetten and coworkers [19] assume, on the contrary, that the bound fraction is more actively resynthesized. In their view, this fraction is bound with protein enzymes, in part phosphorylase. Thus, the formation and breakdown of glycogen proceeds by way of the bound form. This is in agreement with the evidence of Rozenfel'd, who found that binding of glycogen to proteins favors its phosphorolysis. This is also in agreement with the position taken by Merrick and Meyer [21] on the basis of studies of glycogen in heart muscle under conditions of anoxia. In their opinion, free glycogen is converted into bound glycogen during its breakdown; and bound glycogen is accessible to the enzymatic mechanism responsible for glycolysis. On the other hand, Przylecki [22], Breitburg [23], and other authors assume that free glycogen is more easily split by enzymes.

A survey of these various studies is sufficient to convince oneself how little has as yet been clarified in the question of the physiological role of glycogen fractions. Therefore, we do not consider it possible to give any physiological interpretation of the phenomenon which we detected. At the same time one must take into account two studies, which seem to us to have some relevance to our experiments. From the evidence of Khaikina and Goncharova [24], an excited state in the brain favors accumulation of the bound form of glycogen. Gabrielescu [25] used a histochemical method and came to the conclusion that the protein-bound glycogen fraction increases in the sympathetic cervical ganglion during excitation.

However, it is necessary to emphasize that comparison of our results with the studies just cited, as well as with all those mentioned above, is rendered difficult by, among other things, the circumstance that all authors concerned with the question of glycogen fractions studied changes in two fractions, the free and bound fractions, while in our experiments the free fraction is practically nonexistent. It is not present in the organ of Corti, or if it is present in a small quantity, then it does not change during insonation. One cannot exclude the possibility that free glycogen is broken down during isolation of the cochlea, but even if this is so, this would not alter the significance of the basic fact which we established. According to our evidence, one must distinguish between two fractions of desmoglycogen itself, according to the firmness with which they are bound to some tissue component. During insonation the less firmly bound sub-fraction decreases, while the more firmly bound sub-fraction increases. Questions as to the division of desmoglycogen into separate fractions and their changes under the influence of one or another physiological factor have not yet been studied, as far as is known to us. To solve such a question, and also to obtain a more profound understanding of our findings, further experiments are necessary.

CONCLUSIONS

The organ of Corti contains ca. 35 μg of glycogen, or approximately 10-15% by weight. All or almost all the glycogen in the organ of Corti is in the bound form. It cannot be extracted by cold TCA. Most of it can be extracted by TCA only at the boiling point. However, even after such an extraction procedure, some glycogen remains in the precipitate. To extract this especially firmly bound glycogen, the tissue must be treated with hot concentrated alkali.

When the intact animal has been subjected to intense acoustic stimulation for an hour, it is not possible to establish any changes either in the total glycogen content of the organ of Corti or in the separate glycogen fractions.

When the isolated cochlea is insonated, there are still no noticeable changes in the total glycogen content, but the glycogen fraction extracted by hot TCA is distinctly reduced. At the same time, the fraction which is especially firmly bound to the tissue, and is extracted only by hydrolysis of the tissue with concentrated alkali, increases. During insonation, glycogen in the organ of Corti evidently becomes more firmly bound to some tissue components.

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SEPARATION OF THE LECITHINASE AND COLLAGENASE ACTIVITIES OF THE Cl. perfingens TOXIN BY ELECTROPHORESIS ON STARCH

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The toxin of the principal causative agent of gas gangrene, Cl. perfringens, has a complicated antigenic composition. The presence of several toxins in filtrates of gas gangrene cultures was established some time ago [1].

Priggle [1] separated two fractions of the toxin by fractionation with $(NH_4)_2SO_4$ and Na_2SO_4: a fraction possessing a pronounced lethal and a slight hemolytic effect and a fraction with both a hemolytic and a lethal effect. MacFarlane and Knight [2] have presented evidence to the effect that the lecithinase found in toxic filtrates of Cl. perfringens is identical to the alpha toxin. The existence of unstable hemolysin in the toxin was known as early as 1925 [1]. Nell conducted experiments to determine hemolytic activity in a phosphate buffer, in which the hemolytic activity of the alpha toxin, which is activated by Ca^{++}, was totally inhibited and the activity of the theta toxin, a hemolysin, was manifested.

Unfortunately, there are not many works concerned with the separation and purification of toxins and the preparatory isolation and subsequent study of their different fractions, although several physicochemical methods are now known which make it possible to separate proteins differing as to isoelectric point and electrophoretic mobility (preparatory electrophoresis) and which permit considerable purification of the isolated fractions (ion exchange resins and cellulose).

Haberman [3] separated lecithinase, or the alpha toxin, the ϑ toxin, hyaluronidase and a gelatinolytic factor from a filtrate of a culture of Cl. welchii, type A, strain BP6K, by precipitation with methanol, pH 6.5. To separate the activities, the author used electrophoresis on starch with pH 9.0; to purify the fractions, he used adsorption on hydroxyl apatite with subsequent elution by a phosphate buffer (pH 6.80, the elution increasing in ionic strength, and also chromatography on ion exchange resins.

The purpose of our investigation was to fractionate the Cl. perfringens toxin by electrophoresis on starch and to make a biochemical study of certain biological activities of the toxin, namely those of lecithinase, collagenase, proteinase and the hemolytic factor.

METHODS

The toxin of Cl. perfringens, series 796, was obtained by cultivating Cl. perfringens, strain BP6K-28 on a medium based on casein acid hydrolyzate. The native toxin, which contained 2000 lecithovitellin units (LV) per

1 ml or 2 UF* was concentrated by precipitation with 60% $(NH_4)_2SO_4$, dialyzed for two days against tap water and for one day against distilled water in the cold and then lyophilized. One hundred to five hundred mg of dry standard toxin was dissolved in the proper buffer solutions and subjected to electrophoretic fractionation on a starch block [4].

In each fraction, we determined the protein content according to Lowry [5], lecithinase activity [4], collagenase activity according to the splitting of procollagen isolated from rat's skin, expressing it in ctg α [6], homolytic activity by the method proposed by Vygodchikov for titrating staphylococcic hemolysin [7] and proteinase activity according to milk clotting [6]. We used the least quantity of the toxin which would cause almost total hemolysis of sheep's erythrocytes for the minimal hemolytic dose (HE).

RESULTS AND DISCUSSION

Fig. 1 shows an electrophoregram depicting the distribution of the activities of Cl. perfringens toxin fractionated in a boroborate buffer, pH 8.6, $\varkappa^{**} = 0.0035^{-1} \cdot cm^{-1}$. As the graph shows, the collagenase and lecithinase activities of the toxin can be sparated by electrophoretic fractionation on a starch block in a boroborate buffer, pH 8.6. The electrophoretic mobility was $1 \times 10^{-5} \times cm^2 \times sec^{-1} \times v^{-1}$ for lecithinase and $5 \times 10^{-5} \times cm^2 \times sec^{-1} \times v^{-1}$ for collagenase. During electrophoresis, the toxin was largely purified of inert substances such as the proteins of the culture medium and bacterial cell. Because the toxin was considerably inactivated by the action of the current on the proteins, the degree of purification could not be expressed in units of activity per 1 mg protein in these experiments. Neither the original toxin nor any of its isolated fractions possesssed proteinase activity, as assayed by milk clotting. On the basis of our experiment with fractionation of toxin and anatoxins on paper [8], thus separating the toxin and the anatoxins into 3-4 protein components, we undertook in our subsequent work electrophoretic fractionation of the toxin on a starch block in an acetate buffer, pH 5.3, $\mu = 0.1$. Preliminary experiments carried out in order to determine the effect of the type of buffer used on the lecithinase and collagenase activities of the Cl. perfringens toxin showed that both enzymes are more stable in an acetate buffer, pH 5.3, than in the boroborate buffer.

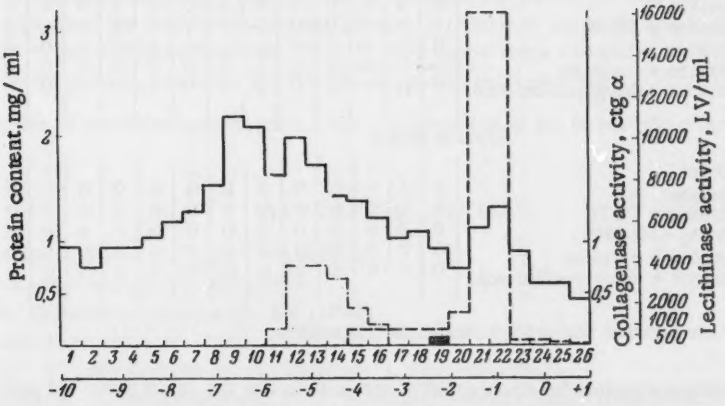

Fig. 1. Electrophoretic distribution of the lecithinase and collagenase activities of Cl. perfringens toxin, fractionated in a boroborate buffer. Shown along abscissa axis: length (cm) of starch block (toxin fractions) and scale of electrophoretic mobility. Solid line shows the protein content in mg/ml; dotted line shows lecithinase activity in LV/ml; dash-dot line shows collagenase activity in ctg α; black rectangle is the application place of the toxin.

*UF—unit of fixation, i.e., the least amount of toxin which will fix one antitoxic unit of the corresponding antiserum.

**$\varkappa$—electrical conductivity.

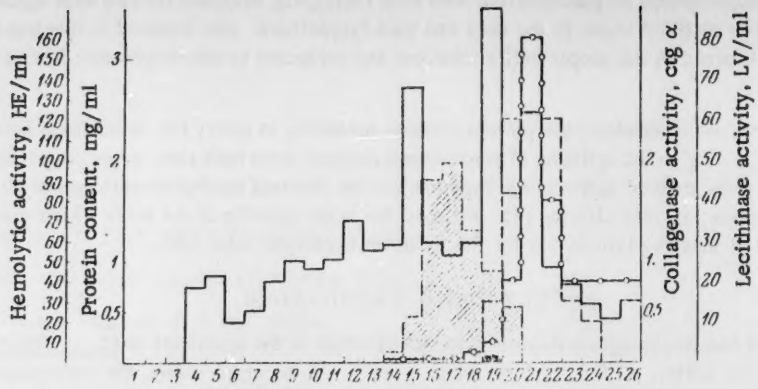

Fig. 2. Electrophoretic distribution of lecithinase, collagenase and hemolytic activities of Cl. perfringens toxin with fractionation in an acetate buffer. Dotted line with circles shows hemolytic activity in HE/ml; other symbols are the same as in Fig. 1.

A Study of the Toxin's Lecithinase, Collagenase and Hemolytic Activities

Experimental materials	Lecithinase					Collagenase					Hemolysins				
	Versene concentration, %														
	0.5	1	2	3	4	0.5	1	2	3	4	0.5	1	2	3	4
Boroborate buffer															
Original toxin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Toxin + versene	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0
Toxin + versene + $CaCl_2$	+	+	0	0	0	+	+	+	0	0	+	+	+	0	0
Toxin + $CaCl_2$ + versene	0	0	+	0	0	+	+	+	0	0	+	0	0	0	0
Toxin + versene + cysteine	+	0	0	0	0	+	+	0	0	0	0	0	0	0	0
Toxin + versene + thioglycollic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetate buffer															
Original toxin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Toxin + versene	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Toxin + versene + $CaCl_2$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Toxin + $CaCl_2$ + versene	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Toxin + versene + cysteine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Toxin + versene + thioglycollic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Note: + = preparation active; 0 = preparation inactive.

Fig. 2. depicts curves showing the distribution of the protein and the lecithinase and collagenase activities of Cl. perfringens toxin along the length of the starch block. The graph shows that separation of the lecithinase and collagenase activities of the toxin and their preparative isolation also occurs under these conditions. This graph also shows the hemolytic activity of the toxin, the mobility of which coincides with that of the toxin's lecithinase activity. Van Heyningen has reported data to the effect that the different strains of Cl. welchii contain 13 factors, which each exert a different physiological and biochemical effect on the live organism. For example, the main component of the Cl. perfringens toxin, the alpha toxin, possesses a lethal, necrotic and hemolytic effect; the beta toxin is lethal and necrotic in effect, and the gamma toxin, lethal. In certain cases, manifestation of this or that effect of the effect of the toxin cannot be associated with the properties of one fraction, but is due rather to admixtures of another fraction, particularly the alpha toxin, which has various biological activities. To prove our proposal, we set up a series of experiments in which lecithinase was blocked by E.D.T.A. (Versene) fixation of the Ca^{++} ions required for the enzyme's action. For this purpose, we added 100 mg E.D.T.A. in a 5% concentration to 2 ml of the original toxin and each active fraction. The inhibitor was used in such a high concentration on the basis of Moscowitz's data [9], which demonstrate that 5% Versene protects a mouse for

two hours from the Cl. perfringens toxin, while a 2.5% concentration only provides 30-minute protection. The inhibition of the toxin's effect is evidently not due to inactivation of the toxin, but to fixation of the Ca^{++} ions required for the manifestation of the lethal effect of the alpha toxin, or lecithinase. We determined the lecithinase, collagenase and hemolytic activities in all the toxin samples with blocked lecithinase, just as in the samples without the added E. D. T. A., and established that all the activities were inhibited.

In order to confirm the role of Ca^{++} in the inhibitory effect of E.D.T.A. and to determine the optimal E.D.T.A. concentration, we performed a series of experiments in which the toxin's activity was blocked by different concentrations of E.D. T.A. in acetate and boroborate buffers, in the presence and absence of $CaCl_2$. The effect of cysteine and thioglycollic acid on E.D.T.A.-blocked Cl. perfringens toxins was also studied. The results of the experiments are given in the Table.

In a boroborate buffer, 0.5% E.D.T.A. inactivated the lecithinase and hemolysins of the Cl. perfringens toxin, but not the collagenase. In an acetate buffer, the collagenase was inactivated while the lecithinase and hemolytic activities were retained. The addition of $CaCl_2$ restored the lecithinase and hemolytic activities blocked by 0.5% E.D.T.A. The addition of $CaCl_2$ to toxin inhibited by 2% E.D.T.A. restored the activity of the enzymes in a boroborate buffer. Reactivation of the Cl. perfringens toxin under the influence of $CaCl_2$ did not occur when the collagenase, lecithinase and hemolytic activities of the toxin were blocked by the addition of E.D.T.A. in higher concentrations. The addition of cysteine to the blocked toxin in amounts equimolecular to the amount of E.D.T.A. added restored to a negligible degree the lecithinase activity of the toxin, while the hemolytic and collagenase activities were observed to be inhibited regardless of the type of buffer in which the blocking was carried out. On the basis of these data, we came to the conclusion that the presence of Ca^{++} is also required for the manifestation of the hemolytic activity of the Cl. perfringenes toxin.

Therefore, the method we used did not succeed in separating the lecithinase and hemolytic activities of the toxin.

SUMMARY

The method of electrophoresis on a starch block in a boroborate buffer, pH 8.6, and in an acetate buffer, pH 5.3, was used to separate the lecithinase and collagenase activities of the toxin and to determine the electrophoretic mobility of the analogous fractions. The Cl. perfringens toxin and its fractions obtained by electrophoresis on starch do not possess proteinase activity, as assayed by milk clotting.

Calcium ions are of essential importance for the manifestation of the hemolytic effect of the Cl. perfringens toxin.

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ON THE PROBLEM OF THE BIOSYNTHESIS OF THE PYRROLIDINE RING IN NICOTINE

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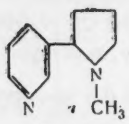
The biosynthesis of alkaloids is closely connected with the nitrogen metabolism of plants. The belief is widely held that plants synthesize alkaloids from amino acids and their conversion products [1]. Experimental endeavors on this subject, utilizing isotope methods, have yielded particulary valuable results [2].

The question of the biosynthesis of nicotine—the main alkaloid of the tobacco plant—occupies a special position in this problem.



Nicotine

The nicotine molecule incorporates into itself the important heterocycles—the pyridine and pyrrolidine rings, which are the basic structural units of many alkaloids, certain amino acids, major coenzymes, and a number of vitamins and hormones. In addition, the biosynthesis of nicotine is linked with the process of methlation of the N-atom. Analysis of this process is of considerable interest in connection with the study of the synthesis of such substances as choline, and betaine, which play an important role in the processes of transmethylation. All this illustrates the importance of resolving the problem from a general biological point of view, and explains the interest of many investigators in its study.

Contemporary efforts to study the biosynthesis of nicotine have been carried out on whole plants or on sterile cultures of tobacco roots.

We used tobacco sprouts as the subject of the investigation. In a previous work we established the interrelation between the metabolism of proteins and amino acids and the biosynthesis of nicotine. It was shown that along with the appearance and subsequent accumulation of nicotine in the tobacco sprouts there also occur significant changes in the amino acid composition (especially in the concentration of bound and free glutamic acid and arginine).

On the basis of the results obtained it was postulated that a number of amino acids serve as the forerunners of nicotine in the tobacco sprouts [3]. For the final settlement of this question the investigation was continued, using tagged compounds.

METHOD

Seed germination technique. Five grams of the seeds of Nicotiana tabacum, Trapezond 93 variety, were sown on filter paper in Petri dishes. Into each dish we introduced 30 ml of Knop's nutrient solution in a 1:2 dilution. The covered Petri dishes were transferred to humidifiers for germination (the sprouts were watered as necessary). The experiments were carried out at a temperature of 20–22°. In order to provide optimal growth conditions we employed supplementary illumination with daylight lamps (12 hours a day). Under these conditions the sprouts normally grew and synthesized nicotine. We did not observe the development of microorganisms.

Introduction of the tag. The following amino acids were used as tagged compounds: 1-C^{14}-glycine, 2-C^{14}-glycine, C^{14}-methionine, C^{14}-leucine, C^{14}-glutamic acid, and C^{14}-arginine. To each Petri dish we added an amount of one of these compounds corresponding to 30 millicuries, bearing a specific activity equal to 2.6 millicuries/gram (the specific activity of these compounds was adjusted by the addition of a vehicle). The tagging compound was added along with the Knop's nutrient solution on the 1st, 2nd, 3rd and 4th day of germination, in allocations of 15, 5, 5 and 5 millicuries respectively. The introduction of the tagged amino acids did not have any effect on the normal growth of the sprouts.

Analytic methods. The nicotine was extracted from the sprouts on the 6th, 8th and 10th day following the onset of germination. The exposure time was selected in accordance with the data of Schmid and Serrano [4], which established that the most active synthesis of nicotine occurs in the first 10 days of germination of the seeds. At the end of the experiment the sprouts were carefully ground in a porcelain mortar with the addition of quartz sand. The nicotine was distilled from the ground material with water vapor. The acidified distillate was concentrated in a water bath to a volume of 2–2.5 ml. The alkaloids were precipitated with picric acid. Precipitation of the dipicrates of nicotine was carried out by cooling with ice for 10–12 hours. The dipicrates coming out were removed by filtration, washed in iced distilled water and recrystallized. The purity of the nicotine preparations obtained was judged by the form of the crystals (characteristic yellow spicules) and by their melting point, 217–218°. Portions of the dipicrates of nicotine in the range of 2–5 mg were used for the determination of their radioactivity.

RESULTS AND DISCUSSION

Data on the concentration of the alkaloids and their specific radioactivity are presented in the table.

We did not observe sharp fluctuations in the concentration of the nicotine isolated from the sprouts within groups of the same duration of exposure. The minimal difference which we observed may have been caused by unavoidable variations in the development of the individual sprouts. This made a comparison of the specific activities of our preparations acceptable.

Radioactive nicotine was extracted in our experiment in all cases, but the total radioactivity of the alkaloids isolated from the sprouts was relatively low. With germination of the seeds into sprouts there occurs active hydrolytic processes leading to a sharp elevation in the quantity of free amino acids [3]. In conjunction with this it is possible that when the tagged compound is added it may be diluted considerably. In addition, at those

Quantity of Nicotine and Its Specific Radioactivity

Amino acids administered to the sprouts	Nicotine, mg			Specific radioactivity, imp/min/millimole×10^3		
	6*	8	10	6	8	10
1-C^{14}-glycine	0.9	5.9	9.9	2.4	2.0	2.0
2-C^{14}-glycine	1.4	6.8	9.7	1.8	2.7	2.5
C^{14}-methionine	0.9	7.0	10.0	2.9	6.1	1.7
1-C^{14}-leucine	1.4	7.0	11.0	7.0	5.8	1.5
C^{14}-arginine	1.4	6.9	11.1	5.5	8.3	1.1
C^{14}-glutamic acid	1.2	5.9	9.9	6.9	12.7	6.1

* Numbers in this series – age of the sprouts in days.

intervals of exposure employed the nicotine might be actively involved in the general metabolism of the sprouts [4].

The noteworthy part of nicotine in the metabolism of the tobacco plant has been demonstrated in recent times by the works of a number of authors. Thus, Bose [5] isolated an enzyme from Nicotiana tabacum which, in the presence of a methyl acceptor—ethanolamine, catalyzed the conversion of nicotine to nor-nicotine. Therefore, it was finally proven that the N-methyl group of the alkaloid is capable of taking part in the reactions of transmethylation. The work of Tso and Jeffery [6] demonstrated the significant changes which are undergone by nicotine, tagged at the ring and at the methyl group, with its introduction into the intact plant. In this case the greatest reduction in activity of the nicotine was observed by the authors in the first days following the administration of the tagged compound, with small subsequent decrease in activity, and the appearance of the isotope tag in other compounds.

Leete and Bell [7] produced radioactive choline and showed that nicotine acts as the donor of the methyl group in the tobacco plant.

As can be seen from the table, the sepcific radioactivity of the nicotine, extracted at varying intervals of germination from the sprouts that were treated with the same compound, did not remain the same. In this case, the most intensive incorporation of 1-C^{14}-glycine and 1-C^{14}-leucine occurred in the 6 day old sprouts; when we used 2-C^{14}-glycine, C^{14}-methionine, C^{14}-arginine or C^{14}-glutamic acid this was seen in the 8 day old sprouts. Taking into account the varying rate of incorporation of these compounds into the nicotine molecule, we subsequently compared the specific radioactivites of the preparations obtained at their maximum level.

Ranking by intensity of incorporation, the amino acids may be listed in the following descending order: glutamic acid, arginine, leucine, methionine, 2-C^{14}-glycine, 1-C^{14}-glycine. The data on the active incorporation of arginine present special interest. There is no evidence in the literature concerning the part of this amino acid in the process of nicotine biosynthesis. Possibly, preliminary conversion of the arginine to ornithine occurs in the sprouts via one of the reactions in the ornithine cycle: arginine $\xrightarrow{\text{arginase}}$ ornithine + urea. The amino acids: C^{14}-methionine, 2-C^{14}-glycine are potential sources of methyl groups in the biosynthesis of nicotine; C^{14}-methionine stands out among them.

In order to compare the results we obtained with the data in the literature, we briefly devoted ourselves to studying the biosynthesis of the pyrrolidine ring in nicotine, and the process of its methylation. In recent times a number of investigators have shown the active participation, in the synthesis of the pyrrolidine ring, of the system: arginine—putrescine—proline—glutamic acid, the so-called "glutamic acid series." An interrelationship has been shown between the amino acids within this "series," and the possibility of their interconversion has been proven [2]. In the case of nicotine, synthesis of the pyrrolidine heterocycle is realized by this pathway. Thus, independent of each other, Leete [8] and Dewey et al. [9] showed that in the biosynthesis of nicotine 2-C^{14}-ornithine is the compound most intensively incorporated into the molecule. Using supplemental feedings of 1-C^{14}-glutamic acid on the tobacco plant, Lamberts and Byerrum [14] produced radioactive nicotine. In this case, 10% of its activity was noted in the pyridine ring, and ~ 90% in the pyrrolidine ring.

Dewey [11] observed the incorporation of 2-C^{14} leucine in the pyrrolidine ring of nicotine. The question of methylation in the biosynthesis of nicotine was studied in detail by Byerrum et al. [13-18]. The authors were able to demonstrate the incorporation of the isotope from the following radioactive compounds into the N—CH_3 group of nicotine: C^{14}-forming acid [12, 13], C^{14}-formaldehyde [14], β-C^{14}-serine [14], $C^{14}H_3$-methionine [12, 15], $C^{14}H_3$-choline [15], $C^{14}H_3$-betaine [16], α-C^{14}-glycolic acid [13,17], and also 2-C^{14}-glycine [18]. Analysis of the results obtained by the authors already mentioned shows that methylation of the N-atom of the pyrrolidine heterocycle may occur via two pathways:

1. Through the use of glycine, glycolic acid, and serine the oxidative cleavage of a single carbon component occurs, with the formation of formaldehyde. This appears to be carried out by folic acid [2]. Subsequently the formaldehyde is reduced to a methyl group.

2. When methionine serves as the source of a methyl group, the methyl group is transported as such, without preliminary oxidation, i.e., the typical transmethylation reaction occurs. This fact was proven by an experiment using methionine which was doubly tagged by CH_3 group [19]. In this case a different group of enzymes participates, which is activated by the addition of ATP. Since the incorporation of formaldehyde into the mo-

lecule of nicotine occurs with the fastest rate, it may be postulated that in plants the methylation of the N-atom in the pyrrolidine ring of nicotine is accomplished primarily by the first route.

Summing up, it may be stated that the results obtained by us from the study of the biosynthesis of nicotine in sprouts is, on the whole, analogous to the data obtained from experiments on adult plants. In order to resolve the problem of localizing the carbon from the tagged amino acids in the nicotine molecule it is necessary to further study the products of oxidation from the preparations of the alkaloid which are obtained.

SUMMARY

Ranking the compounds tested on tobacco sprouts by the intensity of incorporation of their isotope into the nicotine, they may be listed in the following descending order: C^{14}-glutamic acid, C^{14}-arginine, 1-C^{14}-leucine, C^{14}-methionine, 2-C^{14}-glycine, 1-C^{14}-glycine.

When feedings were made with 1-C^{14}-glycine and 1-C^{14}-leucine the maximum specific radioactivity of nicotine was observed in the 6 day old sprouts. In the case of 2-C^{14}-glycine, C^{14}-arginine, C^{14}-methionine, and C^{14}-glutamic acid, this was seen in the 8 day old sprouts.

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AN ELECTROMETRIC METHOD FOR THE STUDY OF TISSUE RESPIRATION

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Warburg's manometric method for the study of tissue respiration has certain disadvantages which limit its widespread use in biochemical investigations.

The cumbersome and complicated manometric technique, as well as the necessity for observing a strict temperature regime, make this method laborius [1, 2]. In addition, its sensitivity is inadequate when working with numberous subjects, since the rate of oxygen absorption by the tissues optimal for recording is 100-150 μl per hour. The minimal volume of the fluid phase absorbing the oxygen requires the use of small quantities of tissue with high activity. As a rule, Warburg's method cannot be used where there is a low respiration intensity or when the work is being done on very small amounts of tissue. Elevating the sensitivity by increasing the length of time the tissue respiration is recorded also doesn't always lead to the desired results, since in this case the air-tight seals of the containers are often disturbed. The use of microrespirometers [3] only complicates the cumbersome manometric technique. Finally, constant agitation of the containers during the incubation is injurious to certain cells [4].

At the same time, there are data in the literature on the electrometric determination of the oxygen concentration in biological fluids, performed directly, using various electrodes [5]. This makes it possible to discard the gasometric method, and to carry out a study of tissue oxygen requirements by altering its concentration in the solution. Using this principle, Harris and Barclay [4] developed a new electrometric method for the study of cellular respiration in macrophages. However, for tissues, especially those with a low respiratory activity, this method, as well as the proposed construction of the chamber, are inadequate. In addition, certain details of the technique are absent from this work [4]. The latter makes this new and highly valuable investigative method difficult to reproduce.

From all this, the necessity arose for further development of an electrometric method for the study of tissue respiration that could be used on the usual tissue subjects. It was also of interest to compare the data obtained from the manometric and electrometric methods.

EXPERIMENTAL SECTION

METHOD

The electrometric method for studying tissue respiration uses only the oxygen dissolved in the fluid phase; in contradistinction to the manometric method, the gas phase is absent. The change in the oxygen concentration of the solution containing the tissue homogenate under study serves as an index of the respiration intensity. The oxygen concentration is determined by using electrodes mounted in the chamber.

Electrodes. The polarizing electrodes consisted of platinum wires, 0.4 mm in diameter, or small disks. The platinum wire or disk was welded to a copper wire (0.8 mm in diameter) and sealed in a glass tube 70 mm long, so that the projecting free portion of the platinum wire was 5 mm, and the free portion of the disk—0.2 mm^2.

The insulated copper wire passed out the other end of the sealed glass tube; it was connected to the positive terminal of the M-95 microammeter.

The non-polarizing electrode was a piece of lead (10 × 4 mm), fused to a copper wire. The latter, at a distance 20 mm from the lead, was covered with a layer of organic glass, and served as the connection to the negative terminal of the apparatus. This electrode was connected with the solution under study via an agar bridge. For the preparation of the agar bridges we used glass tubes (5 × 90 mm), contracted at one end to a bore diameter of 0.3-0.5 mm. The tubes were filled to 20 mm from the narrow end with a 3% agar suspension, prepared in a 1.14% solution of KCl. After their preparations, the agar bridges were kept in the 1.14% KCl. It was possible to use them after two days, but preferably after 5-7 days, following their preparation. Otherwise, the calibration curves were unstable.

Before the procedure, the free end of the tube over the column of agar was filled with 0.1 N acetate buffer, pH 4.6. The lead electrode was inserted into the open end of the tube, submerged in the buffer, and fastened in an immovable position. Following the completion of the procedure, the buffer was removed and the agar bridge again placed in the 1.14% solution of KCl. The lead electrode was always kept in the acetate buffer, and the platinum electrode, in distilled water. Before the procedure and after its completion, the latter was carefully washed in water and rubbed with an ether-soaked wad of cotton. The current intensity was measured on the M-95* microammeter; it ranged from 0-20 μamps. The copper wire connecting the electrodes to the apparatus was insulated throughout its entire length, and its ends, making contact with the terminals, were plated or carefully stripped.

Chambers. Since the tissue respiration was accomplished by using the oxygen in the solution, the construction of the chamber had to guarantee an essentially perfect seal and it had to be possible to completely displace all the air when it was filled. In this work we used two types of chambers, constructed by ourselves.

The first type of chamber (Fig. 1), and the simplest, was a glass test tube (2.5 × 6.0 cm) in which was inserted a rubber stopper with the electrodes mounted upon it. In the stopper there were a total of three openings: two were for the electrodes and one, for a glass tube projecting 1 cm above the stopper. A rubber tube, of corresponding diameter and 2 cm in length, was fitted to the glass tube. After the test tube was filled, the stopper with the electrodes was inserted in it and the remaining air was removed through the open aperture in the stopper by tilting the tube gently from side to side. When the chamber was sealed, the fluid inside was raised through the glass tube to the point where it met with the rubber tube. After a 10-minute temperature equilibration at 37°, leading to expansion of the fluid and a corresponding elevation of its level (already within the rubber tube), 2-3 drops of immersion or vaseline oil were layered on the surface to prevent diffusion of air into the fluid. In this case the level of the fluid in the rubber tube had to be lower than the level of the acetate buffer in the agar bridge, since otherwise the agar might have peeled off and the buffer mixed with the contents of the chamber. After the completion of the procedure the rubber tube, containing the oil, was taken off first, and only then was the stopper with the electrodes removed. This prevented soilage of either the electrodes or the experimental specimen with oil, allowing the specimen to be used for subsequent investigations.

The second type of chamber (Fig. 2) was a modified Warburg vessel. It was usually mounted on the base of the Warburg manometer, which made it possible, in certain experiments, to agitate the vessel during its incubation in the water bath. In this case, 3-5 glass beads were placed in the bottom of the chamber for better mixing. There were three openings in the upper portion of this chamber, 4-5 mm in diameter. Two of these were for the introduction of the electrodes, while the third narrowed to capillary diameter and ended in a tube with a spherical dilatation and a glass stopper. The latter was used to secure the attachment of the chamber to the base. After temperature equilibration, oil was introduced into the spherical dilatation, just as it was in the rubber tube of the first type of chamber. With the aid of the glass stoppers and vacuum grease, the electrodes and the outlet tube to the third opening were tightly joined to the chamber, guaranteeing its essentially perfect seal.

The volume of the chamber was determined gravimetrically; it was 25 and 10 ml respectively for the chambers of the first and second types.

Calibration of the Electrodes. With immersion of the electrodes in the solution and closure of the circuit a polarizing current arises which gradually decreases and, after 1-2 minutes, attains a stable level. The current

*The M-95 microammeter is manufactured by the "Vibrator" Factory in Leningrad.

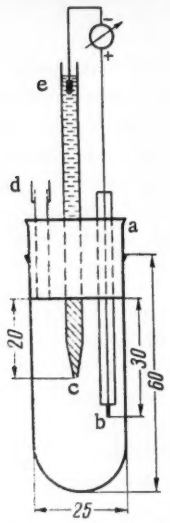

Fig. 1. Schema of the first type of chamber. a) Stopper containing the electrodes; b) platinum electrode; c) agar bridge; d) tube leading to the chamber; e) lead electrode.

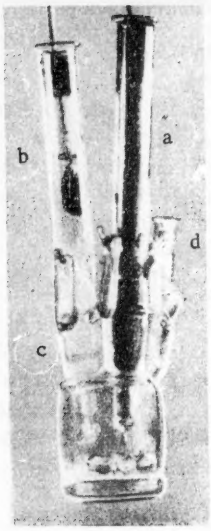

Fig. 2. Full view of the second type of chamber. a) Platinum electrode; b) lead electrode; c) agar bridge; d) spherical dilatation and tube leading to chamber.

intensity is directly proportional to the concentration of oxygen in the solution and the surface of the platinum electrode [5]. If the latter is constant, then the current intensity is proportional to the logarithm of the oxygen concentration [4]. Thus, calibration is done by measuring the current intensity in solutions with known concentrations of oxygen. The oxygen concentration can be determined by various methods [4, 5], the most favorable of which is the technique based on Henry's Law. In this case one can use either the vacuum method [4] or the method of saturating the solution with a gas mixture of known composition [5].

Calibration by the vacuum method was carried out in the following manner. Hanks' solution was poured into a 0.5 liter Bunsen flask so that the fluid layer was 2 cm deep. In the procedure we used a somewhat modified Hanks' solution which did not contain phenol red and $CaCl_2$, since the latter depresses tissue respiration [7]. The pH of the solution was adjusted to 7.4 by the use of 1 N NaOH. Then the Bunsen flask, connected to the vacuum pump and the manometer, was transferred to the water bath and kept at 37° while the gas was removed from the fluid by the vacuum over a period of 3-5 minutes. Following this, the electrodes were placed in the Bunsen flask. The flask was closed with a rubber stopper through which the wires passed that connected the electrodes to the microammeter. The stopper was sealed in place with paraffin. Since the dimensions of the lead electrode do not effect the current intensity, it was possible to carry out the simultaneous calibration of several platinum electrodes (up to 4) with one lead one. Then, at normal atmospheric pressure, the Bunsen flask was agitated in the water bath for 30 minutes. Next the flask was fixed in an immovable position and the current intensity was measured 1 or 2 minutes after closure of the circuit. Analogous measurements were also made at 90, 180, 360, 520 and 660 mm Hg, observing strict standarization of the other experimental conditions.

Calculation of the oxygen concentration in the solution based on its corresponding current intensity was done on the basis of Henry's Law. Henry's constant for oxygen for the levels of 0 to 60° was computed on the basis of data on its solubility in water [8], and then it was determined for 37° by interpolation; it was found to equal 3.94×10^7. The oxygen concentration was calculated in the following manner. The air pressure in the system in mm Hg (P_b) is expressed by the formula: $P_b = P_a - (P + 47.1)$, where P_a is the atmospheric pressure, P is the electrical discharge, and 47.1 is the pressure of saturated water vapor at 37°. The oxygen concentration in the air is 20.95 volumes percent (vol. %), from which the partial pressure of oxygen Po_2 is equal to $0.2095\ P_b$. According to Henry's Law, the molar portion of oxygen in the fluid $N^f_{O_2}$ is determined by the expression $N^f_{O_2} = P_{O_2}/K$. Since the molar concentration of oxygen in the solution C may be taken to equal $55.5\ N^f_{O_2}$, then:

$$C = 55.5 \frac{P_{O_2}}{K} = \frac{55.5 \times 0.2095}{3.94 \times 10^7} P_b \text{ moles } O_2/\text{liter}$$

$$C = 0.295\ P_b\ \mu\text{moles } O_2/\text{liter or } C = 6.608\ P_b\ \mu\text{liter } O_2/\text{liter}.$$

It must be noted that in this calculation we did not take into account the decrease in the oxygen solubility and the decrease in the pressure of the water vapor on the solution as compared to the pure solvent, due to the insignificant magnitude of these corrections.

The electrodes in the chambers of the second type were calibrated by the method of saturation of the solution with a gas mixture of known composition [5]. In contradistinction to the first method, calibration of the electrodes was carried out directly in the chamber itself. To do this, a gas mixture was prepared out of atmospheric air and nitrogen, containing oxygen in the concentrations: 2.1, 5.2, 10.5, 15.7, and 21.0 vols. %. Then the Hanks' solution in the chamber was saturated with each gas mixture by bubbling the gas through the solution for 15 minutes at 37°. After saturation, the electrodes were inserted into the chamber and stoppered in place, the capillary opening was sealed with oil, and, after 15 minutes (necessary for the final temperature equilibration), the current intensity was measured.

It should be noted that complete displacement of the oxygen from the solution by bubbling nitrogen through it under the conditions of the ambient atmosphere is practically impossible. In addition, the usual nitrogen supplied in tanks contains a total of 0.6–0.7% oxygen. Thus, in the calibration it is impossible to obtain the so-called "zero point," which, at any rate, is not particularly necessary.

Calculation of the oxygen concentration in the solution for the second method of calibration was carried out in the following manner. The partial pressure of the oxygen on the fluid P_{O_2} is expressed by the formula:

$$P_{O_2} = \frac{P_a \times C_1}{100},$$

where C_1 is the concentration of oxygen in the gas mixture in vols. % (for air, C_1 = 20.95%). The absorption coefficient of oxygen in water at 37°, arrived at by interpolation, is equal to 0.024 [8]. From this, the oxygen concentration in the solution C is determined by the expression:

$$C = \frac{0.024 \times 1000}{760} P_{O_2} = 0.0316 P_{O_2}\ \mu\text{liters } O_2/\text{ml}.$$

On the basis of the data for the calibration of each platinum electrode, we constructed a curve representing the dependency of current intensity on the oxygen concentration. The working concentration of oxygen usually consisted of 0–180 micromoles/liter. Corresponding to the data in the literature [4, 5], with calibration by the latter method a linear proportion was observed between the current intensity and the oxygen concentration, while with calibration by the vacuum method this linear proportionality was between the current intensity and the logarithm of the oxygen concentration, or, to be more exact, lg (C + 100). The calibration curves are rather stable, although after 1–2 months it is necessary to check them.

Both methods yield, to an equal degree, adequate and coinciding results, but the vacuum method is less difficult.

Determination of the Intensity of Tissue Respiration. The newly developed method was tested on homogenates of the liver and renal cortex of the rabbit, as well as on homogenates of embryonal tissue from the chick.

The extracted tissue was homogenized in Hanks' solution in the proportion 1:10 at 0°. Five to ten ml of refrigerated Hanks' solution was introduced into the chamber, having first been maintained for 30 minutes in a water bath at 37° with periodic agitation for the removal of any supersaturating gas from the solution. Then the chamber was placed in a small bath containing ice, and a definite amount of homogenate was added to the solution (25–100 mg of kidney tissue) plus more Hanks' solution to bring the volume to its final level. The electrodes were inserted into the chamber, air bubbles were completely removed, and it was transferred to the water

bath at 37°. After a 10 minute temperature equilibration, and then after regular time intervals, the current intensity was measured. Immediately after the first measurement oil was layered on the surface of the fluid in the open tube of the chamber. Absorption of oxygen was calculated on the basis of the calibration curve appropriate for the volume of the chamber. It was expressed in micromoles or microliters.

In the procedure we used the TS-15M water bath and the Warburg incubator apparatus, maintaining the temperature at the set level with great accuracy (up to 0.02–0.05°). However, such a strict temperature regime is apparently not necessary for this method, since the temperature coefficient is 2.3% per 1° [5]. On the other hand, with manometric measurements the permissable fluctuation interval of the temperature at the various sites of the incubator is 0.05° [1].

Comparison of the Manometric and Electrometric Methods. The manometric and electrometric methods were compared in simultaneous determinations involved with the investigation of respiration intensity for rabbit renal cortex homogenates. A dry weight of 100 mg of tissue per sample was taken; the respiratory activity was expressed in % of the value found by the manometric method. The following results were obtained:

Incubation minutes	Manometric method	Electrometric method	Number of determinations
30	100	88	8
60	100	86	7
90	100	108	5
120	100	156	3

The results of the determinations show that the two methods yield sufficiently coincident values. Deviation in the levels obtained from these methods did not exceed 14% when investigation of the respiration was performed in the first 90 minutes.

However, while in the course of the first hour the electrometric method yielded somewhat lowered results as compared with the manometric method, in the course of the second hour this relationship was reversed. This may be explained by the fact that in the Warburg apparatus the tissue absorption of oxygen during the second hour of incubation was, as a rule, lower than during the first hour, while with the electrometric method the oxygen absorption was carried out more uniformly throughout the entire incubation period (Fig. 3).

For certain experiments it was necessary to agitate the chamber in studying the respiration of the tissue homogenates. For this purpose, several beads were placed in the chamber and a portion of the chambers were incubated without being moved while the other portion were agitated at a constant rate (60–70 oscillations per minute). The results of these trials showed that there were no essential differences and that no elevation in the intensity of respiration was observed with agitation of the chamber. Thus, in contradistinction to the manometric method, agitation of the chamber during incubation is not necessary in the electrometric procedure.

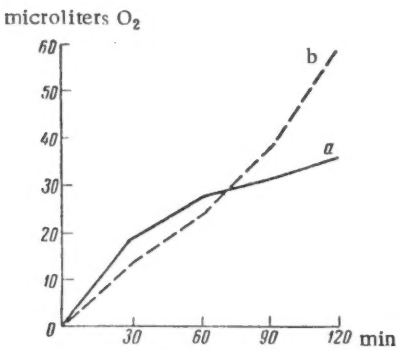

Fig. 3. Respiration of rabbit renal tissue. a) Manometric method; b) electrometric method.

DISCUSSION OF THE RESULTS

The described electrometric method for the study of tissue respiration has several advantages over the manometric method.

The main advantage is the high sensitivity of the new method, which permits recording the absorption of oxygen at a rate of 5–10 microliters/hour. Its sensitivity may be still further elevated by decreasing the volume of the chamber. This method affords the opportunity to work with very small amounts of tissue (up to 5 mg of renal tissue), as well as with a large amount of tissue of low respiratory activity. It is also important that the electrometric method is technically simpler, since it does not require a strict temperature regime and eliminates the need for agitating the chamber.

Further, in investigating respiration intensity and its dynamics the new method yields more uniform results throughout the duration of the experiment. The rate of oxygen absorption, even at the end

of the fourth hour of incubation, does not significantly differ from the first hour, as opposed to the manometric results. The latter fact, plus the elimination of the agitation, permits the use of this method for investigations lasting many hours.

The comparison between the electrometric and manometric methods shows that they yield sufficiently coincident results in simultaneous determinations. However, the enumerated advantages of the electrometric method justify regarding it as more useful in the study of tissue respiration

SUMMARY

A modified electrometric method for the study of tissue respiration was described. This method can replace the Warburg manometric method in certain investigations.

The electrometric method is technically simpler, but more sensitive than the manometric method. In simultaneous determinations the two methods yield sufficiently coincident results.

In conclusion, we would like to express our thanks to Doctor of Biological Sciences I. P. Ashmarin for submitting the theme of this investigation and for his constant guidance in its fulfullment.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. ***Some or all of this periodical literature may well be available in English translation.*** **A complete list of the cover-to-cover English translations appears at the back of this issue.**

DETERMINATION OF FLAVONOID COMPOUNDS AFTER THEIR SEPARATION BY PAPER CHROMATOGRAPHY

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After their separation by paper chromatography, flavonoid compounds may be determined either by the intensity of fluorescence and the size of the spots (semiquantitative analysis) or by the colorimetric method [1] after elution of the flavonoids from chromatograms and electrophoregrams.

A whole series of colorimetric [2-14], spectrophotometric [15, 16], polarographic [17-19] and fluorometric [20] methods of determining flavonoid substances have been described. The disadvantage of all methods used to determine flavonoid substances is their lack of specificity, since whole groups of substances, sometimes substances differing sharply as to physiological nature, give identical or extremely similar reactions. Direct determination of flavonoid substances without their preliminary separation can only be used in the case of certain simple pharmaceutical preparations. When natural mixtures are being analyzed, these substances should first be separated. The method most widely used for this purpose is paper chromatography.

Chromatographic separation of flavnoids on paper was first used by Bate-Smith [21], Wender and Gage [22] et al. [23-25]. Gage, Douglas and Wender [26] quote R_f values for 38 flavonoid substances in 11 systems of solvents. In Czechoslovakian literature, information on the behavior of flavonoids in paper chromatography has been collected by Prochazka [27]. The systems most frequently used in practice are: water-saturated ethyl acetate and n-butyl alcohol–CH_3COOH–H_2O in a ratio of 4:1:5. To detect flavonoids, their own yellow color, their fluorescence or color reactions are used. Bate-Smith and Westall [28] established specific correlations between the structure of flavonoids and the chromatographic behavior in separate chromatography, as defined before the polar phase. Hais [27] has described in detail the behavior of flavonoids in paper chromatography.

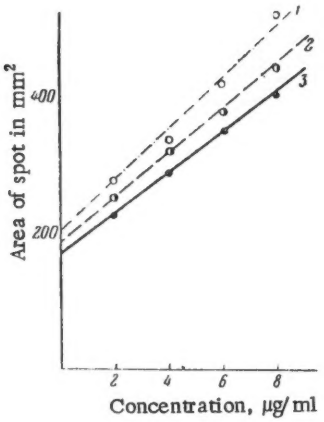

Fig. 1. Calibrated curves depicting the data obtained from chromatography of flavonoids. 1) Naringin; 2) quercitrin; 3) rutin.

Separation of flavonoids by chromatography on a column of various adsorbents such as aluminum oxide, calcium sulfate, kaolin, cellulose, silica gel, etc. [29-31] or on ion exchangers such as Dualite A-2, Dualite A-4 and Amberlite IRC_{50} [32, 33] is used only for preparation because of the non-quantitative nature of such separation.

The method of chromatography on a nylon column has recently been used [34-35]. This method had already been used to quantitatively determine rutin in certain material with a high content of this flavonoid [36, 37]. However, chromatographic separation on paper is a widely used and convenient method for the separation of mixtures with a low content of flavonoid substances.

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EXPERIMENTAL PART

Paper Chromatography of Flavonoids

Colorimetric measurement was performed with a Pulfrich colorimeter (Zais Ena factory) in a cuvette per 1 cm.

Substances Used: Rutin from the Lakhema firm, melting point 184°; quercitrin from the Geidis firm; melting point 176°; naringin from Sunkist Growers, melting point 212°. We used several solvent systems for the separation of rutin, quercitrin and naringin. The most successful was the mixture: n-butyl alcohol–CH_3COOH–H_2O (mixture I, 4:1:5, mixture II, 4:1:2.2). Mixture II was successfully used by Roberts for the separation of tea flavonoids. It provides total separation of rutin, quercitrin and naringin, making it possible to obtain clearly delimited spots.

Separation was carried out by descending chromatography on Whatman No. 1 paper. We used the flavonoids own fluorescence, i. e. the fluorescence which develops after the action of ammonia fumes on the chromatograms (rutin and quercitrin form brownish-yellow spots, naringin forms bright yellow spots), or the color reactions with diazo salts of aromatic bases to detect the substances. Mixture II was used to separate rutin, quercitrin and naringin. Solutions of rutin and quercitrin in methanol and naringin in ethanol were applied to a strip of chromatographic paper; the paper was put in a chromatographic chamber for about ten hours, after which the solvent was passed through it from above for 12 hours. Detection of flavonoids was carried out by both of the methods mentioned above. Clearly defined spots of the different flavonoids appeared on the chromatogram as a result. The R_f values obtained constituted: 0.57 for rutin; 0.84 for quercitrin; 0.71 for naringin.

We next undertook to conduct a semiquantitative analysis of the chromatograms according to both fluorescence intensity and spot size.

Semiquantitative Determination of Flavonoid Concentrations: As established in the preliminary experiments with the use of ultraviolet light to demonstrate the spots, comparatively small differences in the concentration of the different flavonoids cause very large differences in fluorescence intensity and spot size. We therefore attempted to appraise the approximate concentration of flavonoids according to the fluorescence intensity and spot size.

One, two, three or four μg rutin, quercitrin or naringin, in 5 μl of the solvent in every case, was applied to strips of chromatographic paper. Methanol was used as the solvent for the first two flavonoids; ethanol was used for naringin. Therefore, according to the amount of the substance applied to the chromatogram, an ascending order of 1-4 μg was obtained for each flavonoid. Chromatography was performed at room temperature. The solvent used was mixture II, which was passed through from above until the solvent front approached the end of the chromatogram. For detection of the flavonoids, the dried chromatogram was examined in ultraviolet light and treated with ammonia fumes.

These experiments established that the concentration of rutin and quercitrin can be visually evaluated from the spot size and intensity of fluorescence with an accuracy of ±1μg, while the concentration of naringin can be evaluated with an accuracy of ±0.5 μg. One should note that this method of quantitative evaluation is also commonly used to determine other substances, such as riboflavin. This method is valuable for preliminary orientation preceding strictly quantitative determination of flavonoids. On the basis of the concentration thus determined (we determined it approximately), it is very easy to find the batch of specimens required, their concentration, dilution, etc.

Determination of Flavonoids by Paper Chromatography: We next attempted to use paper chromatography for direct determination of flavonoids. For this purpose, we used flavonoid solutions in concentrations of 1, 2, 3 and 4 μg of the flavonoids per 5 microliters methanol (for rutin and quercitrin) or ethanol (for naringin).

The samples were applied with a micropipette so that the diameter of the spot was 0.5 cm (when dried slightly by heating). The various flavonoid specimens were applied in amounts of 1, 2, 3 and 4 μg; mixture II was used for separation. After drying, the chromatogram was subjected to fluorometry and to the action of ammonia fumes, as well as to processing with diazotized p-aminobenzoic acid. The p-aminobenzoic acid solution was prepared as follows: 2 ml 10% H_2SO_4 and 4 ml 0.2% $NaNO_2$ were added to 2 ml of a 0.5% p-aminobenzoic acid solution. The mixture was stirred, 6 ml 10% NaOH added, the whole stirred again and the prepared solution immediately used for development. When this method of development is used, the sprinkling of the

chromatograms should not be excessive, because the spots would wash out, and their exact reproduction would then be practically impossible. The spots demonstrated by fluorometry or by the color reaction were sketched, and their area was measured by planimeter or with the aid of transparent graph paper. The data obtained were used to construct graphs showing the relationship between the size of the spots and the concentration of flavonoids (Fig. 1).

As the graph shows, the relationship is always linear in character, any lines other than straight ones being due to inexact application of the samples or error in determining the size of the spots.

Therefore, this method of separating flavonoids can be used for their quantitative determination, but its accuracy is low, and the results obtained have a rather large range of fluctuation — ± 20%. The advantage of this method is that it permits simulataneous determination of several flavonoids placed closely together — rutin, quercitrin and naringin, for example. The essential disadvantage of the method is the variable nature of the results obtained, which is due chiefly to inaccurate drawing of the spots detected. This method does, however, provide approximate quantitative analysis of flavonoids, the accuracy of which can be increased to a certain degree by the number of analyses.

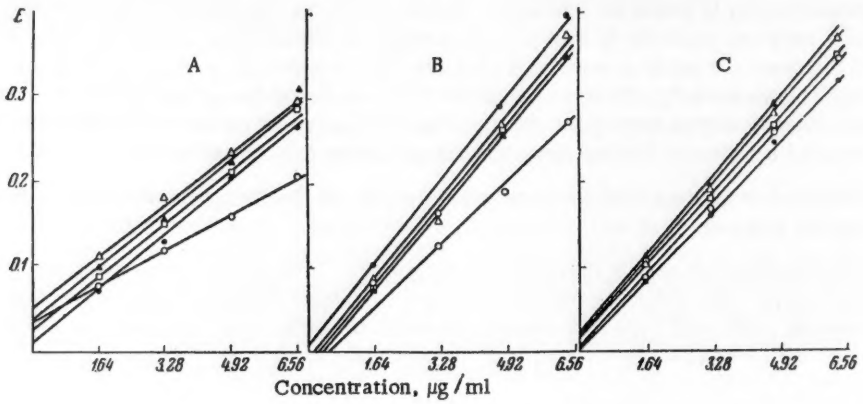

Fig. 2. Progress of flavonoid elution in time. A) Rutin; B) quercitrin; C) naringin. White dots-elution immediately following the reaction; black dots-eluted after 3 hr; squares-after 6 hr; black triangles-after 8 hr; white triangles-after 20 hr.

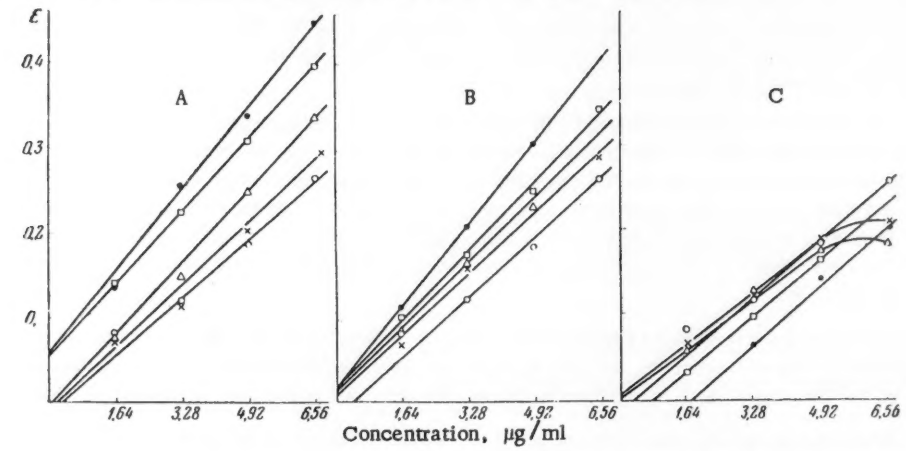

Fig. 3. Effect of acid and alkali on quercitrin elution. Eluting mixture: A) 0.2 N HCl-methanol; B) 0.2 N H_2SO_4-methanol; C) 0.2 N NaOH-methanol. The ratio of 0.2 N acid (or alkali) to methanol was: white dots-0:5; squares-1:4; black dots-1:1; triangles-4:1; crosses-5:0.

Electrophoretic Separation of Flavonoids

The method of paper electrophoresis was used to separate flavonoids (rutin, quercitrin, naringin and hesperidin). Approximately 5 μl naringin in ethanol, 5 μl rutin and quercitrin in methanol and 5 μl of a solution of hesperidin in pyridine were applied with a micropipette to chromatographic paper. The solutions were prepared so that there was approximately 10 μg of the experimental specimen in the volume used. Electrophoresis was performed with a voltage of 360-400 volts. A series of buffers were tested; acetate and phosphate buffers proved unsuitable. The best results were obtained with alkali buffer solutions (especially barbital and borate buffers) in which the above flavonoid substances are easily soluble. In a borate buffer, pH 9.23, we were able to completely separate rutin, quercitrin, naringin and hesperidin in as little as four hours; since these substances in high concentrations have a yellow or orange color, the electrophoregrams obtained did not require development. When lower concentrations were used, the electrophoregrams were developed with the aid of ultraviolet light of diazo salts (see "Chromatographic Separation").

Elution of Flavonoids from Chromatograms and Electrophoregrams

It is clear from the above that separation of flavonoids for analytical purposes can be accomplished only by paper chromatography or electrophoresis. The method of chromatography on nylon should only be used to separate flavonoids in the case of substances in which their concentration is high. In this connection, we next attempted quantitative elution of the chromatograms and developed a technique for colorimetric evaluation of the eluates obtained.

We used Whatman No. 1 paper for separating mixture II. The chromatographic paper was treated with 5, 10, 15 and 20 μl of the flavonoid solution (rutin and quercitrin, in methanol; naringin, in ethanol) with a concentration of 4 μg/μl. Separation was performed by descending chromatography for about ten hours. The spots on the dried chromatogram were located with the aid of ultraviolet light and processing with ammonia fumes. The flavonoid solutions were applied so that the spots were as equal as possible in size. A piece with the same area as a flavonoid spot was taken from a developed chromatogram without a flavonoid to serve as control. Elution was done right in the test tubes. Methanol was found to be the best eluent for rutin and quercitrin, while ethanol proved best for naringin.

The pieces cut from the chromatogram were put into test tubes and 5 ml of the eluent was added. The test tubes were stoppered, and elution was carried out, the test tubes being agitated periodically. Then 0.4 ml 0.5% p-aminobenzoic acid, 0.4 ml 10% H_2SO_4, 2 ml 0.2% $NaNO_2$ and 5 ml 10% NaOH were added to each test tube. The contents of the test tube were thoroughly mixed, and the optical density of the solution was measured--immediately in the test with rutin and quercitrin and after 20 min in the test with naringin, with 420 mμ as compared with the "blank test." The measurement results are shown in Fig. 2.

Fig. 2 shows a slight increase in the degree of extraction as the elution period increased. Total elution was obtained after 8-20 hr. The model experiments established that elution is practically accomplished quantitatively, and that the color reaction product is more soluble than the actual flavonoids. Elution was most complete if the solution was thoroughly shaken after the reaction.

We investigated the effect of H_2SO_4, HCl and NaOH in pure form and mixed with methanol or ethanol on elution of flavonoids. The acids and alkali were used in 0.2 N solutions. Elution was carried out by the method described above in test tubes with an eluent volume of 5 ml. We tested the following eluting mixtures: 0.2 N acid or alkali-methanol (or ethanol) in ratios of 0:5, 1:4, 1:1, 4:1 and 5:0. The color reaction was carried out by the usual method (addition of 0.4 ml 0.5% p-aminobenzoic acid, 0.4 ml 10% H_2SO_4, 2 ml 0.2% $NaNO_2$ and 5 ml 10% NaOH) after the addition of the eluting fluid. The subsequent determinations were performed as described above. The measurement results are given in Fig. 3.

The figure shows that the most complete elution was obtained in an acid medium. The optimal medium for elution appeared to be the 1:1 mixture of 0.2 N H_2SO_4 (or HCl) and methanol (or ethanol); with this medium, the color reaction could be produced immediately after the addition of the eluent.

DISCUSSION OF RESULTS

Elution of chromatograms or electrophoregrams, carried out by the method described, is especially efficient in combination with the colorimetric method previously described [1]. Under these conditions, flavonoids

which have not passed completely into solution are extracted after the color reaction is carried out, because the reaction product is so easily soluble that a vigorous shaking is all that is required to establish equilibrium between the conjugate adsorbed on the paper and the solvent. The other color reactions formerly used for flavonoids are unsuitable for this purpose both because of their low sensitivity and because of the poor solubility of the complexes developed.

Our method has been successfully used to determine rutin in European elder blossoms.

SUMMARY

The proposed method of determining flavonoid substances after their separation by either paper chromatography or paper electrophoresis is especially convenient for specimens with a low content of these substances. The method permits quantitative determination of the individual flavonoids present in one mixture. The method of quantitative elution is particularly convenient for subsequent colorimetric determination with p-aminobenzoic acid. The method also makes it possible to conduct the color reaction right in the test tubes, since the solubility of the products formed is so high that vigorous shaking after completion of the reaction ensures their practically complete elution. The elution is most complete when the mixture 0.2 N HCl or H_2SO_4 and methanol (rutin, quercitrin) or ethanol (naringin) is used in a ratio of 1:1.

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THE BIOSYNTHESIS OF AMINO ACIDS FROM OXALACETIC AND PYRUVIC ACIDS IN LIVING TISSUES OF GERMINATING AND DEVELOPING GRAIN

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In [1], we demonstrated the intensive synthesis of aspartic acid and alanine from oxalacetic acid, which occurs in extracts of wheat and barley sprouts. The synthesis of these amino acids is attended by the consumption of asparagin, glutamine, gamma-aminobutyric acid, glutamic acid and serine, which are used up in the process of transamination with oxalacetic acid and the pyruvic acid formed by the decarboxylation of the latter.

The purpose of this work was to investigate the biosynthesis of amino acids from oxalacetic and pyruvic acids in living plant tissues.

METHOD

Barley sprouts and developing wheat-ears were used as the objects of our study. Oxalacetic acid (OAA) was synthesized by the method of Wohl and Oesterlin [2], with account taken of the observations of Gruber et al. [3]. Pyruvic acid (PV) was purified and converted into its sodium salt according to Price and Levintow [4]. Ammonium salts were obtained by neutralizing OAA and PV with ammonia to pH 7.0.

Part of the experiments were conducted with eight or nine-day-old barley sprouts grown on filter paper, on tap water under natural lighting conditions. By vacuum-infiltration, 0.1 M solutions of the sodium and am-

TABLE 1. Content of Amino Acid in Barley Sprouts Infiltrated with OAA (in μmol/g dry weight)

Experiment No.	Type of experiment	Aspartic acid	Alanine	Aminobutyric acid	Glutamic acid	Glutamine
1	Water	13.1	27.5	20.4	11.9	28.7
	OAA-Na	28.9	35.4	17.0	19.1	31.1
	OAA-NH_4	13.1	47.2	30.6	11.9	43.1
	NH_4HCO_3	13.1	35.4	13.6	19.1	33.6
2	Water	10.3	27.8	10.7	15.0	18.8
	OAA-Na	24.9	37.1	8.0	26.2	20.7
	OAA-NH_4	12.4	52.5	16.0	26.2	31.7
	NH_4HCO_3	12.4	43.2	8.0	18.7	26.4
3	Water	6.6	37.1	23.5	22.5	18.1
	OAA-Na	16.6	34.6	17.1	19.4	21.1
	OAA-NH_4	6.8	40.0	23.5	18.0	36.2
	NH_4HCO_3	6.8	33.4	23.5	—	27.1

monium salts of OAA or else a solution of NH_4HCO_3, neutralized with HCl to pH 7, were introduced into the green part of the sprouts. The sodium or ammonium salt of PV was also introduced into the tissue in a few experiments. The ammonium salt was obtained immediately before the experiment by neutralizing the PV solution with ammonium bicarbonate. Distilled water was introduced into the control sprouts. After vacuum-infiltration, the sprouts were allowed to stand in the air at room temperature for 2.5 hr in a diffuse light. Then the sprouts were washed with distilled water, dried with filter paper, fixed with boiling ethanol and extracted three consecutive times with 80% boiling ethanol. The combined extracts were evaporated to dryness in a vacuum desiccator at room temperature. The dry residue was dissolved in 10% aqueous isopropyl alcohol. The amino acids and amides in this solution were quantitatively determined. Batches of sprouts were taken after infiltration to determine the content of dry matter.

Determination of amino acids and amides was carried out on buffered paper by the quantitative chromatography method described by Kretovich and Uspenskaya in [5]. We used the No. 2043 filter paper of the Schleicher-Schull firm. The paper used to determine alanine, glutamine and gamma-aminobutyric acid was buffered with a 0.067 M phosphate buffer, pH 6.2. o-Cresol, saturated with the same buffer, was used as the solvent. One-dimensional descending chromatography was performed, the solvent being passed through twice. The paper used to determine serine, glutamic acid and aspartic acid was treated with a 0.067 M NaOH-phosphate buffer, pH 12. Phenol, saturated with the same buffer, was used as the solvent.

To quantitatively determine asparagine, we used a combination of acid hydrolysis and quantitative paper chromatography of the aspartic acid formed. A lateral strip containing a spot of asparagine was cut from the undeveloped chromatogram (the paper was treated with the 0.067 M NaOH-phosphate buffer, pH 12, and phenol, saturated with this buffer, was used to help establish the localization of the asparagine spots on the main part of the chromatogram. The pieces of paper corresponding to the asparagine spots were cut out of the chromatogram and hydrolyzed in sealed ampules with 2 N HCl for two hours on a boiling water bath. The scraps of paper were separated by centrifugation, and the hydrolysis was evaporated to dryness over KOH in a vacuum desiccator; the residue was twice redissolved in water and evaporated in a vacuum over KOH, after which the dry residue was redissolved in 80% ethanol. We determined the aspartic acid content in this solution by the method of paper chromatography mentioned above.*

In the experiments with sprouts, keto acids were determined by chromatographically separating their 2,4-dinitrophenylhydrazones. One ml of a freshly prepared saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl was added to 0.5 ml of the basic solution. The solution was extracted four times by 1.5 ml lots of ethyl acetate (the last extraction should decolorize the solution). The layer of organic solvent was evaporated to dryness at room temperature, and the residue was redissolved in 1 ml 96% ethanol. The 2,4-dinitrophenylhydrazones of the alcohol extract were chromatographed on Schleicher-Schull No. 2043 paper treated with the 0.067 M phosphate buffer, pH 6.2; the solvent used was the mixture proposed by Zappi [6]: n-butyl alcohol-ethanol-water (41:9:50). The 2,4-dinitrophenylhydrazones of OAA, PV and α-ketoglutaric acid were prepared as indices.

TABLE 2. Content of Amino Acids in Sprouts Infiltrated with OAA and PV (in μmol/g dry weight)

Amino acids	Water	OAA-Na	OAA-NH_4	PV-Na	PV-NH_4	NH_4HCO_3
Aspartic acid	6.8	11.3	6.8	9.0	8.0	6.8
Alanine	40.4	37.0	47.2	50.5	60.6	33.7
Asparagine	56.7	56.7	73.8	85.2	56.7	102.3
γ-Aminobutyric acid	23.3	20.4	23.3	20.4	20.4	20.4
Glutamic acid	15.3	22.5	21.4	17.6	16.3	15.3
Glutamine	20.6	12.4	24.7	16.4	26.7	26.7

*A method developed in collaboration with Z. S. Kagan at the A. N. Bakh Institute of Biochemistry of the USSR Academy of Sciences, Moscow.

TABLE 3. Content of Amino Acids in Developing Wheat-Ears after the Introduction of Salts of Oxalacetic Acid (in μ mol/g dry weight)

Development stage	Experimental variant	Aspartic acid	Alanine	γ-Aminobutyric acid	Glutamic acid	Glutamine	Serine
Milk	H_2O	1.68	6.62	2.69	3.21	3.56	1.55
	OAA-Na	3.22	7.79	2.36	3.59	3.09	1.55
	OAA-NH_4	3.75	9.74	2.69	3.40	11.87	1.85
	NH_4HCO_3	3.22	7.79	2.19	2.83	5.71	1.55
Beginning of wax	H_2O	3.17	5.53	1.71	4.21	1.22	1.98
	OAA-Na	4.23	6.72	2.22	3.83	1.22	2.12
	OAA-NH_4	6.34	9.48	2.22	5.94	9.64	2.12
	NH_4HCO_3	3.70	5.53	1.71	4.31	6.99	2.24

RESULTS

The results of the experiments with living sprouts infiltrated with the ammonium and sodium salts of OAA are given in Table 1.

The data in the table show that the introduction of OAA-Na into living plant tissue is followed by intensive synthesis of aspartic acid, attended by the consumption of γ-aminobutyric acid, evidently the source of the amine groups in the process of transamination with OAA. Therefore, it is evident that OAA is actually the non-nitrogenous precursor of aspartic acid in living plant tissue. At the same time, our data indicate that the metabolism of aspartic acid is closely associated with the metabolism of γ-aminobutyric acid. This fact, which we established in an earlier work with extracts [1], like the data which indicate the close interconnection of the metabolism of γ-aminobutyric acid in the metabolism of the plant organism.

We did not observe any accumulation of aspartic acid in sprouts infiltrated with OAA-NH_4. The reason for this is that, in the presence of ammonium ions, the aspartic acid synthesized is immediately used for the synthesis of asparagine. This fact is clearly apparent from the data in Table 2.

In the experiments in which the ammonium and sodium salts of OAA were vacuum-infiltrated into barley sprouts, just as in the experiments with extracts, we observed the synthesis of alanine, which was particularly energetic when OAA-NH_4 had been introduced into the tissues. Alanine is formed by the amination of the pyruvic acid formed by the decarboxylation of OAA. The decarboxylation process is, in all probability, chiefly explained by the action of β-decarboxylase, the presence of which in plants has been demonstrated by Vennesland and Felsher [8]. A certain part of the PV, however, is formed by the spontaneous decarboxylation of OAA. An extremely energetic synthesis of glutamine was observed with the introduction of OAA-NH_4 into the tissues, and considerable accumulation of glutamine was observed in all the experiments.

TABLE 4. Content of Amino Acids in Developing Wheat-Ears after the Introduction of PV Salts (in μmol/g dry weight)

Development stage	Experimental variant	Aspartic acid	Alanine	γ-Aminobutyric acid	Glutamic acid	Glutamine	Serine
Milk	H_2O	1.68	6.10	2.69	3.21	3.09	1.55
	PV-Na	1.68	7.4	2.69	4.15	2.14	2.11
	PV-NH_4	3.22	11.0	2.36	4.15	9.50	1.55
	NH_4HCO_3	3.22	5.84	2.36	2.65	7.60	1.55
Beginning of wax	H_2O	3.17	5.53	1.88	4.21	1.45	2.12
	PV-Na	3.60	7.71	1.38	3.45	0.96	2.12
	PV-NH_4	4.87	16.88	1.71	5.17	6.26	1.88
	NH_4HCO_3	3.60	5.13	1.54	4.41	6.26	2.68

Therefore, our data indicate the OAA-NH_4 is an excellent initial material for the synthesis of glutamine. One should also mention that the paper chromatograms of the keto acid 2,4-dinitrophenylhydrazones showed the ammonium salt of OAA, when introduced into the tissues, to be consumed considerably more rapidly in the sprouts than the sodium salt. OAA is used up much faster for the synthetic requirements of the plant in the presence of ammonium ions. The introduction of PV salts into living sprouts caused a considerable accumulation of alanine in the tissues, and the presence of ammonium ions greatly enhanced the synthesis of alanine from the pyruvate (Table 2). This fact, which was established earlier by Kretovich and Bundel' in experiments with colorimetric microdetermination of alanine [9], is of particular interest. On its basis, one can conclude that alanine, as well as glutamic acid, glutamine, aspartic acid and asparagine, plays a very important role in the plant's assimilation of ammonium salts and in the transformation of inorganic forms of nitrogen into organic.

The next series of experiments were performed with developing wheat-ears at the milk or beginning of the wax stage of development. Field-picked plants (brought to the Institute in wet paper) were divided into the suitable number of lots, using material as uniform as possible. Twenty ears were used in each experimental variant. The lower part of the stem was cut off (above the top internode) under water. Then the ears were put stem-first into 0.1 M solutions of the ammonium or sodim salts of OAA and PV. These solutions were prepared immediately before the experiment by neutralizing OAA and PV with either $NaHCO_3$ or NH_4HCO_3. The control ears were put into distilled water. One sample was put into a solution of NH_4HCO_3, neutralized by HCl. Each batch of ears was put into 50 ml of one of the above solutions. Absorption of the solutions was carried out in the light for eight hours, after which the ears were quickly separated into individual spikelets; the stem was removed, the spikelets were carefully intermixed, an average sample of 15 g was taken and all the batches were simultaneously placed in a Dewar flask with dry ice to be frozen. The remaining ears were used to determine moisture content. Then the frozen samples were ground up in a mortar cooled with a mixture of ethanol and dry ice. The frozen powder obtained was transferred to an Erlenmeyer flask, and a five-fold amount of alcohol was added so that the final alcohol concentration was 80%. The samples were allowed to steep for several days, then the alcohol was filtered out, and extraction was repeated with 80% boiling ethanol. The filtrates obtained after three extractions were combined in a 200 ml measuring flask, and 80% ethanol was added up to the mark. The extracts thus obtained were kept in a refrigerator and used to determine free amino acids.

The results of the experiments with the introduction of OAA salts into wheat-ears are given in Table 3. It is evident from these data that aspartic acid was as easily synthesized from OAA in the developing ear as in the sprouts. It is important to note that aspartic acid synthesis from OAA-NH_4 was considerably more intense than that from OAA-Na. There was simultaneous energetic synthesis of alanine, which, as we have said, forms from the amination of the pyruvate resulting from the decarboxylation of OAA. The data in Table 3 also show OAA-NH_4 to be an excellent initial material for the biosynthesis of glutamine. In this respect as well, therefore, the developing ear is similar to the sprouts.

The results of the experiments with the introduction of salts of pyruvic acid into the ears are given in Table 4. These data show that alanine formed easily from the PV introduced into the ear, and that the processes of alanine synthesis were considerably enhanced in the presence of ammonium ions, just as in the case of the sprouts. The data indicating that aspartic acid was easily synthesized from the PV salts, PV-NH_4 especially, is extremely interesting. This fact, also observed in the experiments with living sprouts, is due to the PV carboxylation reaction according to Wood and Workman, which has been demostrated by Vennesland and co-workers [10] in experiments on wheat seeds and by Bandurski and Greiner [11] in experiments on spinach leaves.

SUMMARY

Oxalacetic acid is an initial non-nitrogenous substance used for the synthesis of aspartic acid in green barley sprouts and developing wheat-ears. In the developing ear, synthesis of aspartic acid from oxalacetic acid is clearly enhanced in the presence of ammonium ions. The difference in the effect of ammonium on aspartic acid synthesis in the sprouts and its effect on that in the developing ear is because sprouts contain large amounts of asparagine, while an ear contains almost none. In the presence of ammonium, therefore, aspartic acid does not accumulate in the sprouts, because it is immediately amidated and transformed into asparagine.

When oxalacetic acid is introduced into the sprouts and ears, its decarboxylation occurs, forming pyruvic acid which is used for the synthesis of alanine. The biosynthesis of alanine from both oxaiacetic and pyruvic acid is sharply enhanced in the presence of ammonium ions. In both the developing ears and the sprouts, therefore, alanine is the compound by means of which exogenous ammonium entering the plant is bound.

Pyruvic acid is an initial substance for the synthesis of aspartic acid, especially in the presence of ammonium ions; this synthesis is accomplished by carboxylation of the pyruvate, followed by the formation of oxalacetic acid.

The introduction of ammonium into the ears causes glutamine accumulation. The biosynthesis of glutamine is considerably enhanced, however, if ammonium is introduced in the form of oxalacetic or pyruvic acid salts. This is because the end product of pyruvic and oxalacetic acids in the Krebs cycle is α-ketoglutaric acid, the raw material for the synthesis of glutamic acid and, from this, of glutamine.

Glutamine, in the developing ear, and glutamine and asparagine, in the sprouts, both bind ammonia and serve as a source of amino- (and possibly amido-) groups in the transamination reactions with oxalacetic and pyruvic acids.

We noted a close interdependence between the enzymatic reactions of the cycle of di- and tricarboxylic acids and the processes biosynthesizing amino acids and amides in plants.

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ISOLATION FROM EGG ALBUMIN OF A FRAGMENT CONSISTING OF A POLYSACCHARIDE AND ASPARTIC ACID

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Although glycoproteins do not occupy the last place by far among proteins as far as their diversity and biological importance is concerned, the chemical structure of these compounds has been investigated very inadequately; furthermore the problem in regard to the bond formed by polysaccharide prosthetic groups with the peptide chains has for all practical purposes not been investigated at all. Only a few brief preliminary communications on the subject that were published quite recently can be found in the literature.

J. W. Roseaver and E. L. Smith [1] report that they have isolated from the enzymatic hydrolyzate of human gamma-globulin a glycopeptide of the following structure:

$$\begin{array}{c} NH_2 \\ | \\ \text{glut.-glut.-asp.-tyr.-glut.-asp.-polysaccharide} \end{array}$$

Aminopeptidase splits off from this glycopeptide all the amino acids with the exception of aspartic acid, which apparently forms the connecting link between the peptide and the polysaccharide.

F. Michel and F. Suthaus [2] hydrolyzed cattle gamma-globulin with aqueous triethylamine. The mixture of glycopeptides isolated by them consisted of glucosamine, galactose, mannose, fucose, phenylalanine, glutamic acid, and aspartic acid.

L. W. Cunningham, B. I. Neunke, and R. B. Nuenke [3, 4] hydrolyzed egg albumin with trypsin and α-chymotrypsin. The glycopeptide which these authors succeeded in isolating contained, in addition to mannose and glucosamine, trypsin, aspartic acid, leucine, serine, threonine, and valine.

The probable sequence of amino acids in this glycopeptide is as follows:

$$\begin{array}{c} \text{tyr.-asp.-(leu., threo., ser.)-val.-leu.} \\ | \\ \text{polysaccharide} \end{array}$$

Similar results were obtained by A. Neuberger and co-workers [5] and also by F. R. Jevons [6]. Neuberger points out that after glycopeptide has been subjected to the action of carboxypeptidase one can isolate from the resulting mixture of proteolysis products the carbohydrate component of egg albumin with aspartic acid bound to it.

The glycopeptides of egg albumin are the subject of the present investigation. The work has begun before the communication mentioned above had been published and was conducted to clarify the nature of the link between the polysaccharide prosthetic group of egg albumin and the peptide chain of this protein.

In the present communication we describe for the first time a preparative method for isolating a fragment of the molecule of egg albumin which contains both the carbohydrate component and aspartic acid. This fragment can be regarded as an initial substance for further work aiming at the clarification of the nature of the bond between aspartic acid and the polysaccharide.

METHODS AND RESULTS

Egg albumin was prepared according to the method described by R. A. Kekwick and R. K. Cannan [7]. Recrystallization was carried out 6-7 times. Different preparations of the protein contained on the average 1.8% of carbohydrates, as determined by the orsinol method [8].

Enzymatic hydrolysis of egg albumin. Five grams of the protein was dissolved in 500 ml of 0.1 N HCl (pH= 2), and the solution was heated to boiling. After cooling, 25 mg of crystalline pepsin was added. Digestion was carried out at 37° under a layer of toluene. After 24 hours, 25 mg of additional pepsin was introduced. After a second 24-hour period, the pH of the solution was brought to 7.8 by adding 2 N NH_4OH, whereupon further hydrolysis was conducted with fresh pancreatine for a period of 14 days. The enzyme was added in 15-mg portions every two days. The pH was kept close to 7.8. On completion of the hydrolysis the pH was changed to 4.5-4.7, and the solution was heated to boiling. The undigested proteins coagulated and then were readily separated by filtration through a paper filter. The transparent solution was evaporated in vacuum down to a volume of 100 ml and then filtered.

Acetone fractionation of the hydrolyzate [9]. The hydrolyzate was acidified with 4 N HCl until it had a pH=3 (the glycopeptides precipitate very slowly from a neutral or very weakly alkaline solution), and then mixed with four volumes of acetone. After the mixture had been allowed to stand under refrigeration overnight, the precipitate was separated from the transparent mother liquor, diluted with water to 70 ml, and the resulting mixture again acidified, after which the glycopeptides were precipitated again with four volumes of acetone and reprecipitated in this manner several times. The precipitate was dried completely in a vacuum desiccator charged with P_2O_5. The glycopeptide concentrate obtained in this manner is free of salts and also of amino acids and peptides having a R_f higher than 0.5, as established by paper chromatography when the system butanol–acetic acid–water is used.

Purification of the glycopeptides on the cation-exchange resin Zeo-Carb 225.* The resin, of 200-500 mesh, was purified by St. Moor and W. H. Stein's method [10] and then converted into the H^+ form with 1 N HCl, whereupon washing with water to a neutral reaction was carried out. Columns 1.5x25 cm were used.

The acetone concentrate of glycopeptides, obtained by the hydrolysis of 5 g of protein, was dissolved in 4 ml of water and then introduced into the column. Elution was conducted with water flowing at the rate of 10 ml/hour. Two-ml fractions were collected.

The main quantity of glycopeptides that comes out of the column is contained in a front corresponding to 5-6 ml of eluate. Because the glycopeptides leave the column together with HCl that is freed on the resin from the hydrochlorides of amino acids and peptides, several drops of pyridine were added to every test tube in order to neutralize the acid. Carbohydrate-containing fractions that came out of the column with the eluate front were combined, whereupon the solution was acidified to pH=3 and mixed with six volumes of acetone. The precipitate that formed was weighed on drying, and the sugar was determined by the orsinol method. The carbohydrate or, carbohydrate content comprised 34-35% of the weight of the precipitate (without glucosamine). The average yield was 85% with reference to the carbohydrate contained in the initial protein.

The glycopeptides purified by employing the cation-exchange resin gave only one spot in paper chromatography with different solvents when toluidine [11] or iodic acid and benzidine [12] were used for development. No reaction with aniline hydrophthalate took place even at very high concentrations of this reagent, which indicates that no free aldehyde groups are present in the substance.

To determine the amino-acid composition, the glycopeptides were hydrolyzed with 5.7 N HCl in a sealed tube at 105° for 20 hours. The hydrolyzate was dried and the amino acids were separated on paper, using a two-dimensional electrophoretic chromatography method. The relative quantities of amino acids were determined by the method described by K. V. Giri and co-workers [13]. In the calculation the quantity of aspartic acid residues was assumed to be equal to unity. The following results were obtained: aspartic acid, 1.0; leucine, 1.5; serine, 0.6; threonine and glutamic acid, 0.9; glycine, 0.3; alanine, 0.2; valine, 0.1; cystine and glycine traces. With dinitrofluorobenzene the glycopeptides yield only dinitrophenyl-aspartic acid, i. e., aspartic acid functions in all of them as the N-terminal amino acid.**

*This operation was carried out by a method devised in our laboratory by N. A. Kravchenko.

** The determination of the N-terminal amino acids was carried out at our laboratory by the Fellow T'si Tieh-Fang, to whom we extend our thanks.

The homogeneity of the purified glycopeptides was checked by paper electrophoresis in the pH range of 2.2-8.5. The most interesting results were obtained at pH=2.7 in 0.25 N acetic acid (Fig. 1,A). We obtained three zones: I, II, and III. The principal quantity of substance is contained in the zone III, which, as can be easily seen in the figure, is diffused and unsymmetrical. Although the glycopeptides have presumably been completely purified of amino-acids and peptides, the fractional figures obtained for individual amino acids and also the electrophoretic inhomogeneity indicate that the fraction that had been separated was a mixture of glycopeptides. To further simplify their composition, the glycopeptides were subjected to the action of carboxypeptidase (CBP).

Splitting of the glycopeptides with CBP. A 200-mg quantity of glycopeptides purified on Zeo-Carb-225 was dissolved in 25 ml of water. By adding several drops of 1 N NaOH, the pH was brought to 8.0, whereupon 2 mg of CBP that had been dissolved beforehand was added to the solution. Hydrolysis was carried out in the presence of toluene at 38° over a period of four days. Fresh portions of enzyme were added daily. When the action of the enzyme ceased, the solution was acidified to pH=5.0, the toluene was extracted twice with ether, and the ether was evaporated by heating.

The capacity of sugars to adsorb on activated carbon was used to purify the glycopeptides from amino acids that had been split off and from protein [14].

Purification of the glycopeptides on carbon. Powdered activated carbon was sifted through a 100-mesh sieve. Small particles were separated out by decanting a suspension of the carbon in water. The most active centers of the carbon were blocked by using stearic acid [15]. A column 1.6×4.7 cm was charged with carbon and washed with water; then the enzymatic hydrolyzate was passed through it under a pressure of 15-20 cm Hg. The column was washed with water until the ninydrin reaction disappeared completely and the glycopeptides were diluted with 30% ethanol. The fractions containing the carbohydrates were combined and acidified to pH=3; the resulting solution was mixed with seven volumes of acetone. The precipitate was dried and weighed. The carbohydrates, determined by the orcinol method, amounted to 40% by weight. The yield was 74% on the average, with reference to the carbohydrates taken for digestion with CBP.

Figure 1, B shows a typical electrophoresis diagram of glycopeptides subjected to the action of CBP and purified on carbon. Comparison of the two electrophoresis diagrams (Fig. 1, A and B), which were obtained in the same experiment, using approximately the same quantity of substance, indicates that after the action of CBP the quantity of substance that gives the very narrow zone II increases. However, the zone III does not disappear completely.

In one of the experiments, zone II was eluted. Chromatographic separation of amino acids of the hydrolyzate indicated that only aspartic acid is present. It became apparent that CBP splits off from a part of the glycopeptides all amino acids with the exception of aspartic acid, while electrophoresis at pH=2.7 makes it possible to separate this fragment from the unchanged glycopeptides. To check this conclusion, a preparative separation of glycopeptides treated with CBP was carried out, using electrophoresis on a celluose block at pH=2.7 [16].

Electrophoresis of Glycopeptides on a Celluose Block

Celluose powder prepared by partial hydrolysis of chromatography paper was used.

Preparation of the block. The celluose block was made in a transparent plastic dish, 25×7 cm. Absorbent cotton wicks wrapped in cellophane were affixed to the edges of the dish in order to connect the block with the electrode vessels. The required quantity of celluose was mixed with 0.25 N CH_3COOH and the mass, having the consistency of a gruel, was poured into the vessel. The block was dried with strips of filter paper placed on its top. After this, the celluose surface was covered with filter paper and glass. Before the substance was introduced, the block had been kept under voltage for one hour. The substance, which had been dissolved in a small quantity of the buffer solution, was placed into a narrow ditch which had been made in the block by means of a glass tube with a tapered tip.

In one of the experiments 18.7 mg. of glycopeptides (7.5 mg with reference to the weight of the carbohydrates) was separated on a block 0.8 cm thick. The electrophoresis was conducted at a potential drop of 7 volts per centimeter and a current strength of 22 milliamperes during a period of 16 hours. After completion of the electrophoresis, the block was dried at room temperature for 2-3 hours and cut into sections of 1 cm each, whereupon each section was eluted with 8 ml of water. Half a milliliter of each fraction was used to carry out

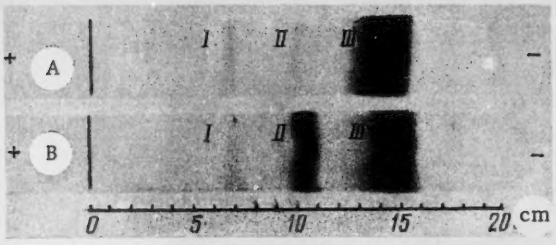

Fig. 1. Electrophoresis diagrams of glycopeptides. A) Glycopeptides purified on Zeo-Carb 225; B) glycopeptides treated with CBP and purified on carbon. Conditions: pH = = 2.7; 0.25 N CH_3COOH soln.; potential 370 volts; duration of electrophoresis 15 hr. Developed with toluidine [11]. Both diagrams were obtained simultaneously in the same experiment. I, II, and II—fraction numbers.

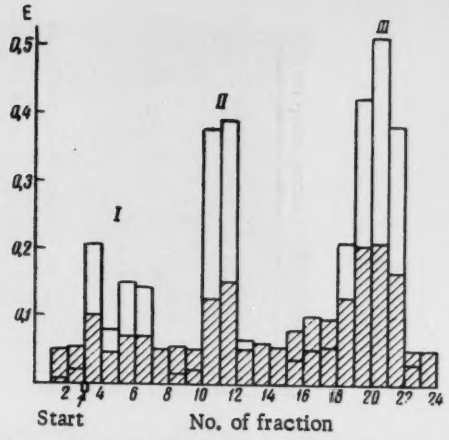

Fig. 2. Electrophoresis on a cellulose block of glycopeptides treated with CBP. Conditions: pH = 2.7; 0.25 N CH_3COOH soln.; potential drop 7 volts per cm ; current strength 22 milliamperes. Duration of electrophoresis 16 hr. The columns that are not cross-hatched represent carbohydrates determined by the orcinol method; the cross-hatched columns represent values obtained by the ninhydrin method.

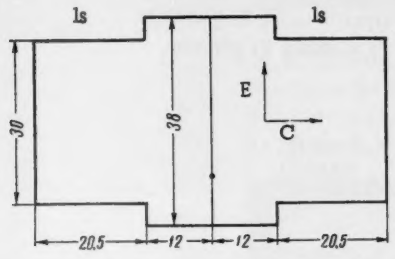

Fig. 3. Prepared sheet of chromatographic paper used in the two-dimensional electrophoresis–chromatography procedure. ls–lateral side of the sheet, which is rolled up on a roller. Here and on the following figures "E" indicates electrophoresis, "C" chromatography.

an analysis for carbohydrates by the orcinol method and 0.5 ml for a ninhydrin analysis according to Moor and Stein [17]. The results are presented in Fig. 2.

The total yield with reference to carbohydrates was 40%. Peak II contained 10% of the total quantity of carbohydrates that had been placed onto the block.

Amino-Acid Composition of Peaks I, II, and III

To separate the amino acids in the glycopeptide hydrolyzates we used monodimensional electrophoresis at pH = 2.7 and also two-dimensional electrophoretic chromatography.

To carry out two-dimensional separations we adapted the commercially available electrophoretic apparatus ÉFA-1 supplied by the Frunze Physical Apparatus Plant. The carrier plate of this device is 25 x 31 cm. A piece having the shape shown in Fig. 3 is cut out of a sheet of chromatographic paper. Precisely through the center of this piece a line is drawn on which the mixture to be analyzed is placed. The lateral sides of the sheet that has been cut out (ls, Fig. 3) are rolled up and kept in a rolled-up state by clamps made of stainless steel. The sheet prepared in this manner is put on the carrier plate of the device, and both the rolls and the paper strip in the middle are sprinkled with the buffer solution; then the clamps are removed, the plate is placed into the apparatus, and a potential is applied. After 30 minutes to 1 hour the mixture to be analyzed is put onto the sheet.

On completion of the electrophoresis one of the rolls is cut off the sheet, whereupon the sheet is spread out and dried. After this, compounds used as testing reagents can be placed on the sheet and chromatographic separation carried out in another direction.

The glycopeptides of peaks I and III were hydrolyzed for 20 hr at 105° in sealed tubes containing 5.7 N HCl. The glycopeptides of peak II were hydrolyzed under the same conditions, but for a period of 12 hours. The peaks I (doubled) and III both contained the same amino-acids: aspartic acid, leucine, serine, threonine, glutamic acid, glycine, alanine, and valine (Figs. 4-6).

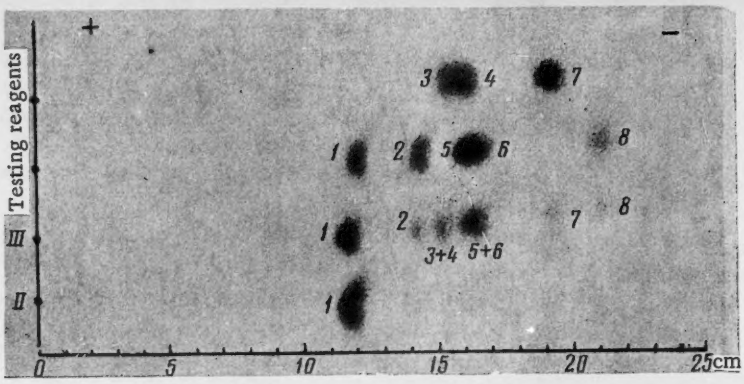

Fig. 4. Monodimensional electrophoresis of hydrolyzates from peaks II and III. pH=2.7; 0.25 N CH_3COOH soln.; potential 1000 volts; duration of electrophoresis 3 hr. Developed with ninhydrin. 1) aspartic acid; 2) glutamic acid; 3) serine; 4) threonine; 5) leucine; 6) valine; 7) alanine; 8) glycine. The glutamine ran off the paper.

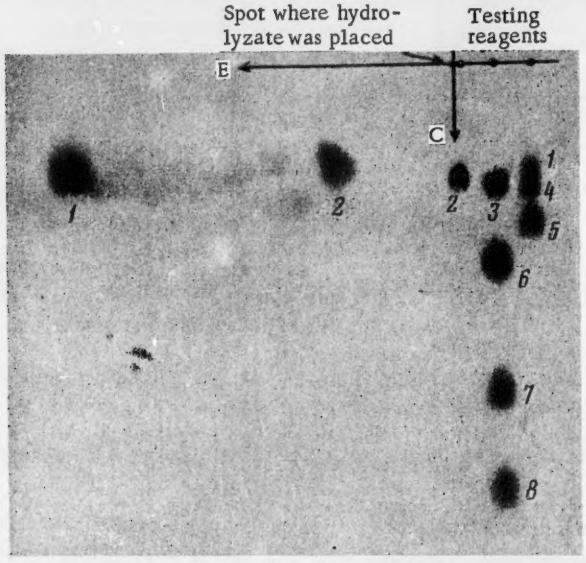

Fig. 5. Chromatographic electrophoresis diagram of peak II. Electrophoresis at pH=2.7 with 1000 volts during a period of 1.5 hr. Solvent: butanol–acetic acid–water (4:1:5).
1) glucosamine; 2) aspartic acid; 3) serine; 4) glycine; 5) threonine; 6) alanine; 7) valine; 8) leucine.

A semi-quantitative determination of the amino-acids of peak III by Giri's method [13] gave the following results: aspartic acid, 1.0; leucine, 1.6; serine, 0.4; threonine and glutamic acid, 0.5; glycine, 0.3; alanine, 0.2; valine, traces. This composition differs little from that of the initial glycopeptide fraction before hydrolysis with CBP had been carried out. In the glycopeptide of peak II, only aspartic acid was found (Figs. 4 and 5).

It has thus been established that aspartic acid forms the link between the polysaccharide and the protein in egg albumin.

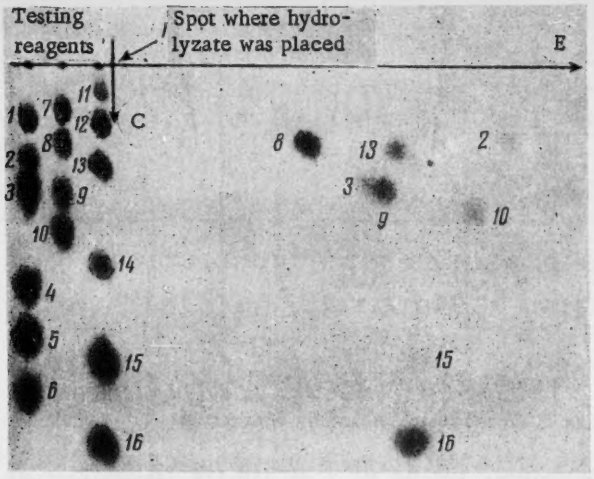

Fig. 6. Chromatographic electrophoresis diagram of peak III. Conditions the same as those in Fig. 5, except that the duration of the electrophoresis was increased to 3 hr. The glucosamine ran off the paper. 1) arginine; 2) glycine; 3) glutamic acid; 4) tyrosine; 5) methionine; 6) phenylalanine; 7) lysine; 8) aspartic acid; 9) threonine; 10) alanine; 11) cystine; 12) histidine; 13) serine; 14) proline; 15) valine; 16) leucine.

SUMMARY

From the products of a thorough hydrolysis of egg albumin with proteolytic enzymes (pepsin and pancreatin), after fractionation with acetone had been carried out and purification on the cation-exchange resin Zeo-Carb 225 in the H^+ form completed, a mixture of glycopeptides could be isolated which was free of amino-acids, peptides, and proteins.

By subjecting the purified glycopeptides to the action of carboxypeptidase and separating the proteolysis products by electrophoresis on a cellulose block, a fragment of the molecule of egg albumin could be separated which contained the polysaccharide prosthetic group of the protein and aspartic acid. This proves that aspartic acid forms the link between the carbohydrate component of egg albumin and the protein peptide chain.

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MECHANISM OF THE OXYGEN EFFECT IN CONNECTION WITH THE IRRADIATION OF PROTEINS

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The oxygen effect, or enhancement of the action of irradiation in the presence of oxygen, constitutes one of the basic problems of radiobiology; it is observed at all levels of radiation activity and takes place everywhere beginning with solutions of proteins and ending with higher organisms. Notwithstanding the great number of investigations which deal with the oxygen effect, the mechanism of this effect cannot be considered clarified at this stage, which is due to a considerable extent to the complexity of the systems subjected to investigation and the indices used for characterizing the effect. For this reason we attempted to investigate the nature of the oxygen effect under relatively simple conditions, i. e., as it is manifested in the oxidation of SH groups of cysteine and egg albumin. This investigation led to some new concepts concerning the nature of the oxygen effect, consideration of which also formed a part of the task to be accomplished by the work described.

EXPERIMENTAL

As object of the investigation chemically pure cysteine and egg albumin that had been recrystallized many times were used. The SH-group content of these compounds was determined by a modified ferricyanide method [1]. Irradiation with x-rays were carried out in a BF tube (8 milliamperes, 160 volts, mean wavelength 0.6 A) with an intensity of irradiation amounting to approximately 10,000 roentgens per minute; irradiation with gamma rays was carried out on a cobalt-60 installation (1,440 g-equivalents of radium) with an intensity of 2,400 roentgens per minute. Irradiation was carried out both in vacuum and in the air and also in a nitrogen atmosphere in equipment described by us earlier [2]. The volume of the gas phase constituted approximately one-half of the volume of the vessel; during irradiation in air contact with the atmosphere was preserved. In some of the experiments dry egg albumin preparations were irradiated.

RESULTS OF THE DETERMINATION

Dependence of the Oxygen Effect on the Dose of Irradiation and the Concentration of the Substrate in Cysteine Solutions. E. Barron and co-workers [3] investigated, on solutions of simple thiol compounds (glutathione and others), the degree of oxidation of SH groups on irradiation in vacuum and in air, finding that at a dose of 5,000 roentgens and a concentration of glutathione amounting to 2×10^{-4}M the yield G on irradiation air equals 10.7, and on irradiation in vacuum, 3.5. However, these results, to which too great a significance has been attached in the literature, apply only under the conditions involved in the experiments in question. We conducted irradiation with different doses and at different concentrations of the substrate (cysteine), establishing that under other conditions results that differ from the values determined by Barron and co-workers are obtained. The data obtained by us are listed in Table 1.

Table 1. Oxidation of SH Groups of Cysteine

Dose (r)	Conc. of cysteine, mols/liter · 10^{-4}	Ionization events per number of SH groups	Cysteine oxidized, mols/liter · 10^{-4}		Oxygen effect (a/b)
			in air	in vacuum (b)	
10^4	8.0	0.03	3.7	1.2	3.08 *
$15 \cdot 10^4$	25.0	0.16	15.0	10.0	1.5
$20 \cdot 10^4$	8.0	0.60	8.0	7.1	1.13
$50 \cdot 10^4$	8.0	1.7	8.0	8.0	1.0
Fenton's reagent**					
in acidic solution	12.5	2.5	12.5	12.5	1.0
in neutral solution	12.5	2.5	12.5	12.5	1.0

* The same results were obtained at irradiation intensities of 500 and 1000 r/min.

** The data with Fenton's reagent (a chemical source of OH-radicals) were obtained at a ratio of OH/SH=2.5.

Barron's data that have been referred to above [3] correspond to a ratio of the number of SH groups to the number of ionization events equaling 14, while the magnitude of the oxygen effect is 10.7/3.5=3.06; this shows good agreement with our data listed on line 1 of Table 1 (a/b=3.08). If we now arrange these data according to the ratio of the number of SH groups to the number of ionization events, a correlation between this ratio and the magnitude of the oxygen effect becomes apparent:

SH/number of ionization events	Oxygen effect (a/b)
30	3.08
14	3.06
6	1.5
1.6	1.13
0.6	1.0
0.4	1.0

With a large excess of the substrate the oxygen effect reaches the limiting value of approximately 3.0; as the excess of substrate is reduced, the ratio a/b gradually drops to 1.0. In other words, when the oxygen effect is estimated in the customary manner by comparing changes on irradiation in air with changes on irradiation in vacuum, one may obtain for the same system data which differ greatly, depending on the ratio of the substrate concentration to the dose of radiation applied; under the experimental conditions indicated in the first two lines a very strong oxygen effect is produced, whereas under the conditions corresponding to the last two lines the oxygen effect is apparently completely absent. These data characterize the uncertainty of the value obtained for the oxygen effect when it is estimated in the customary manner and show that this value depends to a great extent on the conditions under which the experiment has been carried out. It will be seen below that similar data were obtained by us for solutions of egg albumin (see Table 2).

Titration of SH Groups in Solutions of Egg Albumin Irradiated in Vacuum and in a Nitrogen Atmosphere. Aqueous 2% solutions of egg albumin were subjected to irradiation with doses in the range of 51,000-630,000 roentgens. The air was first pumped out of the vessel in which irradiation was to be carried out [2]; an oil pump was used for 20 minutes to accomplish this. After irradiation 0.7 g of guanidine hydrochloride was added to 0.5 ml of the solution. The solution was diluted with water to 1.5 ml, whereupon samples of 0.2 ml were titrated with 5×10^{-4}N $K_3Fe(CN)_6$ [1]. Comparison of results of the titration of solutions that had not been irradiated and solutions that had been irradiated in vacuum (Fig. 1, curve 1) showed that the SH group titer remained entirely unchanged. This result is somewhat surprising, since oxidation of SH groups of the protein with active OH radicals

was to be expected because of the formation of these radicals on irradiation of aqueous solutions in vacuum, especially if one considers that oxidation of this type took place on irradiation of cysteine solutions in vacuum (Table 1). On irradiation of a protein solution in an atmosphere composed of an inert gas (N_2), the titer of the SH groups also remained unchanged (Fig. 1, curve 1); furthermore, the same results were obtained on irradiation of solutions with gamma rays.

Oxidation of SH Groups in Solutions of Egg Albumin Irradiated in Air. On irradiation of a 2% solution of egg albumin with doses in the range of 50,000-630,000 roentgens in the presence of oxygen, in contradistinction to the experiments carried out in vacuum and in nitrogen, oxidation of SH groups did take place to an extent which increased with the magnitude of the dose. The content of unoxidized SH groups on irradiation in air changed as shown by curve 2 (Fig. 1), which is entirely different from curve 1, whereas irradiation of cysteine solutions in air reduced to only a small extent the SH group titer corresponding to irradiation in vacuum (Table 1).

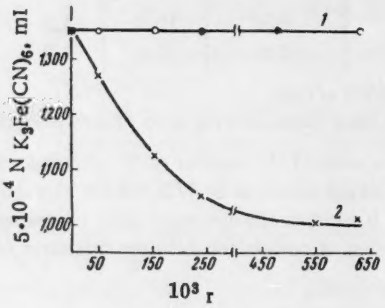

Fig. 1. Change in the thiol group titer of a 2% aqueous solution of egg albumin on irradiation with x-rays. Curve 1: O—before irradiation (mean value of 8 measurements); 1.35 ml of 0.0005 N $K_3Fe(CN)_6$ solution corresponds to 8.2 mg cysteine per 1 molecule of protein); approximately 3 SH groups per protein molecule. O—after irradiation in vacuum; O—after irradiation in a nitrogen atmosphere. Curve 2: X—after irradiation in the presence of air.

It was established in subsequent experiments that the difference between the course of the curves 1 and 2 (Fig. 1) on irradiation of albumin solutions depends to a considerable extent on the partial pressure of air during irradiation. It can be seen from Fig. 2 that at a partial air pressure not exceeding 100 mm Hg, the results do not differ from those obtained in vacuum; whereas at a pressure of 350 mm Hg the same effect is observed as that at 1 atm. If it is assumed that the solubility of air in a 2% protein solution is the same as in water (22.4 mg/l at 760 mm Hg, T=20°, a 34% O_2 content in the dissolved air) approximately 1 molecule of oxygen per molecule of protein must be contained in the solution at a pressure of 1 atmosphere; however, it is possible that this ratio is somewhat higher in protein solutions. If the ratio is reduced to 1 molecule of O_2 per 3-4 molecules of protein (Fig. 2) the results obtained do not differ from those corresponding to irradiation in vacuum.

It is interesting that the marked increase in the action of oxygen, which is expressed in the difference between curves 1 and 2 (Fig. 1), is observed only in connection with the irradiation of protein solutions in the presence of air. When irradiation of the protein solution was carried out in vacuum, subsequent introduction of air into the irradiated solution and letting this solution stand for 2-24 hours did not change the SH group titer as compared with that of a solution on which titration was carried out in the air immediately after irradiation. Because the content of SH groups did not change in the solution irradiated in vacuum, as compared with that in a solution which had not been irradiated, it follows that addition of air after irradiation of a protein solution which is in direct contact with oxygen results in a reinforcement of the oxidation effect.

The Role Played by Denaturation Factors on the Oxidation of Thiol Groups During Irradiation. In all experiments mentioned above, titration of the thiol groups was carried out following addition of 0.7 grams of guanidine hydrochloride to 0.5 ml of the irradiated protein solution. It has already been mentioned that during irradiation in vacuum under these conditions no oxidation of thiol groups of the protein takes place. Very different results were obtained when irradiation of the protein solution was carried out in the presence of guanidine. In the experiments in question irradiation with different doses was applied to a 0.66% solution of egg albumin that contained 4.9 M of guanidine and had been kept for 30 minutes at 37° before irradiation; after irradiation the thiol groups were titrated directly in the irradiated solution. The results of these experiments are shown in Table 2, which is arranged similarly to Table 1 (the 0.66% concentration of egg albumin was selected in order that the molar content of SH groups amounting to 8.2×10^7 moles/milliliter, if five SH groups are assumed to be contained in one molecule of egg albumin [4], should be equal to the concentration of cysteine in the experiments listed in Table 1).

It follows first of all from Table 2 as well as from the numerical data cited above that the magnitude of the oxygen effect depends on the relationship of the substrate concentration to the irradiation dose under the conditions of the experiment. At 50,000 roentgens, when an excess of the substrate is present, the oxygen effect in the protein solutions reaches the same maximum value as in solutions of cysteine (approximately 3.1). As the dose increases and the relative quantity of substrate decreases, the magnitude of the oxygen effect drops and this effect disappears entirely at a dose amounting to 240,000 roentgens.

Table 2. Oxygen Effect in Connection with the Radiation Oxidation of SH Groups in Egg Albumin Solutions

Dose, 10^3 r	Ionization events per ml	Conc. of SH groups in mols/ml	SH groups per ml	SH/number ionization events	SH groups oxidized *		Oxygen effect (a/b)
					in vacuum (b)	in air (a)	
50	$8 \cdot 10^{16}$	$8.2 \cdot 10^{-7}$	$49.2 \cdot 10^{16}$	6.2	0.30	0.95	3.1
100	$16 \cdot 10^{16}$	$8.2 \cdot 10^{-7}$	$49.2 \cdot 10^{16}$	3.1	0.50	1.00	2.0
240	$39.4 \cdot 10^{16}$	$8.2 \cdot 10^{-7}$	$49.2 \cdot 10^{16}$	1.25	1.00	1.00	1.00

* Complete oxidation of SH groups in the solution investigated is assumed to correspond to 1.00.

On the basis of Table 2 the important conclusion can also be made that in the presence of guanidine, even when irradiation is carried out in vacuum, oxidation of SH groups of the protein nevertheless takes place. This oxidation increases when oxygen is present, i. e., the behavior of the protein solution becomes analogous to that of a solution of cysteine, while at a sufficiently high dose of radiation (240,000 roentgens) complete oxidation of the SH groups takes place even in vacuum. Thus, addition of guanidine, which is known to act as an denaturing agent, increases oxidation of SH groups during irradiation and to some extent exerts an effect similar to that of oxygen during irradiation. This effect apparently is due precisely to the denaturing action of guanidine rather than to some kind of secondary reactions, because addition of an irradiated guanidine solution to the protein did not bring about a change of the SH-group titer. The explanation advanced by us is made more plausible by the circumstance that at a weaker degree of denaturation which is due, for example, to acidification of the solution to pH 2.5 (by means of an HCl-KCl buffer solution), irradiation of the protein solution even with a dose of 240,000 roentgens does not change SH-group titer on irradiation in vacuum. When protein is titrated with iodine, the thiol groups are already oxidized completely in natural protein, so that there is no need to denature the protein by adding guanidine, as has been done in cases in which the ferricyanide method was applied. Experiments involving titration with iodine confirmed the results obtained in vacuum and nitrogen at a dose or irradiation equal to about 500,000 roentgens; at a partial air pressure of about 300 mm Hg titration with iodine cause the same reduction of the titer of SH groups which was observed on titration in air.

Oxidation of Thiol Groups on Irradiation of Dry Egg Albumin Preparations. The irradiation was carried out in sealed tubes that had been evacuated or filled with air and contained 70 mg of dry crystalline egg albumin free of any salt content. Irradiation was carried out with gamma rays at doses of 1 and 5 million roentgens. The weighed samples of irradiated protein were mixed with dry guanidine hydrochloride (0.7 grams/10 mg of protein) and dissolved in a phosphate buffer solution (about 0.2 M); the titration of the SH groups was carried out by the ferricyanide method.

At a dose of 1 million roentgens no changes in the titer of SH groups were observed, while at a dose of 5 million roentgens partial oxidation was detected even in the vacuum experiments; this oxidation amounted to approximately 12% of the SH titer in control samples and it increased to approximately 24% when air was present.

DISCUSSION OF RESULTS

The oxygen effect is usually characterized in radiobiology on the basis of the increase of the conversion obtained on irradiation of the system in question in air above the conversion obtained on irradiation with the same dose in vacuum (or on the basis of the ratio of doses when the resulting conversion is the same). It follows first of all from our experiments with cysteine and egg albumine that the magnitude of the oxygen effect determined in

this manner must depend strongly on the relation between the concentration of the substrate and the dose of the radiation applied and that the measured effect is, therefore, essentially relative. With the same system one may, depending on the conditions of the experiment, obtain either the maximum value of the oxygen effect or find that the oxygen effect is totally absent.

Although one can conclude that under the conditions applied in our experiments complete oxidation of the substrate must take place in vacuum (and that, therefore, irradiation in air cannot give a stronger effect), the extent of oxidation in work with living cells and organisms cannot be always evaluated in advance, so that a conclusion in regard to an absence of the oxygen effect or its magnitude may be made without a sufficient basis. In order that the oxygen effect take place to the fullest extent, the presence of a sufficient excess of the substrate is necessary, which is not always the case under the conditions encountered when living cells are irradiated.

It is known that in work with sources which produce dense ionization (alpha particles, etc.), the oxygen effect is not of great importance. This, however, can be explained precisely by the fact that under the conditions obtained in the irradiation, a high local concentration of OH radicals arises, which considerably exceeds the concentration of the substrate. It follows from our data that absence of an oxygen effect can also be observed in irradiation with x-rays, although in this case we have a uniform and rather low volume density of ionization. An apparent absence of the oxygen effect can also be observed when the quantity of substrate is greatly inadequate.

In the case of x-ray irradiation of a cell, the centers of ionization will be located in different sections of the cell so that, notwithstanding the uniform distribution of these centers, there may be more substrate in some parts of the cell than in others. This means that in the same experiment, in different sections of the cell, for instance, sections with a different content of water, the oxygen effect may either be observed or found to be absent, while with the use of other indices of the process (for instance, cell division, oxidative phosphorylation, lipid exchange, etc.) entirely different results are obtained.

These conclusions lead directly to an evaluation of the roles played by direct and indirect action of irradiation in the oxygen effect. In experiments by Barron and co-workers [3] and a number of other investigations [5], the oxygen effect was correlated only with the indirect action of radiation. Reinforcement of the action of radiation in the presence of oxygen is explained from this point of view by assuming that besides OH radicals (which are formed also in vacuum), HO_2 and H_2O_2 are additionally formed and exert a strong oxidative effect. Theoretical calculations by Barron [3] show that at 100 ev up to 12 molecules of glutathione can be oxidized by irradiation of an aqueous solution (which actually amounts to G=10.7), while in the absence of oxygen, when formation of O_2H radicals does not take place, this value drops to 3.5. The ratio of 10.7/3.5=3.08 characterizes the limiting magnitude of the oxygen effect in irradiation of aqueous solutions of simple thiol compounds. As can be seen from Table 1, the limiting magnitude of the oxygen effect in our experiments on the irradiation of aqueous solutions of cysteine amounted to precisely 3.08. Thus, reinforecement of the oxidation of simple thiol compounds during irradiation with x-rays of their aqueous solutions in the air corresponds from the quantitative standpoint to an additional formation of HO_2 and H_2O_2, i. e., it can be fully explained by the indirect effect of radiation.

However, a different situation exists when a protein solution is irradiated. While on irradiation of glutathione in vacuum $G = 3.5$ is obtained, it can be seen from Fig. 1 that oxidation of SH groups of egg albumin is absent under the same conditions, i. e., OH radicals, which presumably exert a strong oxidative action, do not oxidize the thiol groups of the protein at all. On the other hand, a considerable extent of oxidation of SH groups of the protein can be achieved in the presence of oxygen (Figure 1, curve 2); calculation of the ionic yield indicated that it is equal to 1 in this case [1], which corresponds to full participation of both O_2H and OH radicals. It is apparent that these results differ significantly from the data obtained for solutions of simple thiol compounds.

It was shown in a number of published investigations that the oxygen effect connected with the irradiation of proteins can be undoubtedly explained by the direct action of radiation. It has been demonstrated most strikingly by Alexander [6] that on irradiation of dried trypsin with rapid electrons or gamma rays there is an increase of inactivation (by approximately 30%) if irradiation is carried out in oxygen, a phenomenon which this author explains by the formation of either organic peroxide radicals or of the molecular ion O_2^-. The author does not consider these assumptions from the quantitative standpoint; in regard to O_2^- he only points out that this ion may easily inactivate the protein molecule.

One may state that molecular oxygen is actually a strong acceptor of electrons (the energy of the formation of O_2^- amounts to 1.465 ev [7]; however, OH radical are still stronger acceptors of electrons, because the energy

of formation of OH^- comprises 3.7 ev [8]. One may, therefore, expect that irradiation of solutions in vacuum must bring about oxidation of protein SH groups if OH radicals are present: however, as has been shown, this oxidation does not take place, reinforecement of the effect in the presence of oxygen can hardly be explained by formation of O_2^- with which a direct reaction affecting SH groups is associated. Oxidation of SH groups, as well as any other groups, must be accompanied by a loss of electrons, although one may imagine that in a molecule of natural protein this loss of electrons is compensated by an influx of electrons from other atomic groupings, if one accepts the idea in regard to the possibility of the migration of electrons along the protein molecule [9]. From this point of view, the oxygen effect could be explained by the fact that oxygen molecules, by capturing free electrons from other parts of the protein molecule, prevent compensation by them of the loss of electrons in SH groups during oxidation with OH radicals and thus in the final accounting contribute to an oxidation of SH groups. By assuming this, one can also easily explain the difference in the oxidation of SH groups of protein and cysteine, because in a cysteine molecule the compensating transfer of electrons to the SH groups must be absent. Finally, reinforcement of the oxidation of SH groups in vacuum in the presence of guanidine, which has been established in other experiments, can also be explained by a destruction of the network of hydrogen bonds and the resulting interference with the transfer of electrons in the protein molecule, a transfer which is closely associated, according to M. Evans and J. Gergely [9] with preservation of the natural structure of the protein molecule.

However, one encounters difficulties in making this assumption, because the basic concept in regard to the direct migration of electrons or electron excitation along the protein molecule has not been sufficiently proven and has at present met with well-founded objections [10].

An alternative assumption in regard to the formation of free peroxide radicals in proteins as a result of the direct action of radiation can also be objected to. Alexander and co-workers [11] point out that irradiated protein is capable of catalyzing the polymerization of methacrylic acid, although they do not evaluate in any manner whatsoever the absolute quantity of radicals that are formed. An evaluation of this sort was made by applying the direct method of measuring electronic paramagnetic resonance in work carried out by Shêng P'ei-Kêng, L. A. Blumenfel'd, A. E. Kalmanson, and A. G. Pasynskii [12], who established that irradiation of dried natural egg albumin in vacuum with a dose of 2×10^7 roentgens results in the formation of 5.4×10^{17} paramagnetic particles per 1 gram. This result is still more significant because it has been obtained with the same preparation we used and at the same gamma-ray installation which we applied in our work. As has been mentioned above, with a dose of 5×10^6 roentgens applied in vacuum approximately 12% of the total SH groups capable of being titrated in a control experiment were oxidized (which is in close agreement with an ionic yield of 1), so that one may assume that at a dose of 20 million roentgens approximately 48% of the SH groups titrated in the control experiment will be oxidized, which corresponds to the oxidation of approximately 1.5 SH groups per molecule of the protein, whereas 5.4×10^{17} of radicals or unpaired electrons per 1 gram of protein (having a molecular weight of 40,000) give a ratio of 1 radical per 30 molecules of protein. With irradiation in vacuum under the same conditions 45 oxidized SH groups correspond to 1 radical. In the case of irradiation in air this discrepancy becomes still greater, because the number of peroxide radicals (after addition of O_2) according to Alexander is equal to the number of primary radicals, while the number of oxidized SH groups under these conditions increases by a factor of 2. This quantitative discrepancy is so great that direct radiation oxidation of SH groups can hardly be explained by assuming that free radicals form as a result of the scission of hydrogen bonds, because if this were the case one would expect a close correspondence between the number of radicals and the quantity of oxidized SH groups.

For the reason stated, we arrived at a different assumption in regard to the mechanism of the oxygen effect, which in our estimation is in closer agreement with accepted ideas in regard to the structure of protein molecules. As can be seen from Fig. 1 (curve 1), irradiation of aqueous solutions of protein in vacuum with a dose up to 600,000 roentgens does not result in any oxidation of SH groups at all, while in the presence of guanidine (Table 2) approximately 30% of these groups are oxidized at a dose of 50,000 roentgens and 50% of these groups are oxidized at a dose of 100,000 roentgens. It is known that the principal action of guanidine on protein molecules consists in the breakage of hydrogen bonds and that this is the reason why the compound in question exerts a denaturing action. Apparently it is precisely this action of guanidine which results in a reinforcement of the oxidation of SH groups, because no secondary guanidine reactions could be detected in our experiments. The SH groups of natural protein have a lowered reactivity because of participation of the hydrogen atoms of these groups in the formation of hydrogen bonds and other intermolecular bonds. The breakage of these bonds in the presence of denaturing substances may lead to an increased mobility of the hydrogen atoms and reinforcement of the polarity of the SH bonds accompanied by a corresponding increase of the chemical activity of the SH groups [13].

It is important to note that this effect can be produced not only by substances of the guanidine or urea type, but also by molecular oxygen, which because of its electronegative nature may participate in hydrogen bonding and thus bring about redistribution of hydrogen bonds in the protein molecule accompanied by an alteration of the natural structure of this molecule. For example, it can be seen from Fig. 2 and Table 2 that a 30% or 50% oxidation of SH group can be brought about either by irradiation in vacuum in the presence of guanidine or irradiation in the absence of guanidine at an appropriate partial pressure of air; with the same doses of irradiation, but in the absence of either factor the SH groups of the protein are not oxidized at all. On the other hand, in the case of irradiation of cysteine solutions, in which the thiol groups do not participate in the formation of any hydrogen bonds and these groups retain their full activity, noticeable oxidation takes place already in irradiation in vacuum and with a sufficiently high dose of radiation can be brought to completion without resorting to guanidine or oxygen.

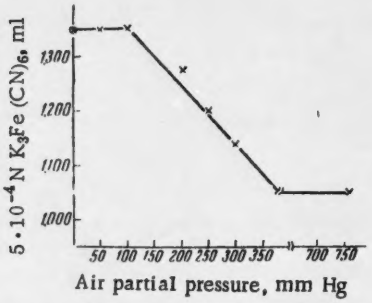

Fig. 2. Change in the titer of thiol groups in 2% aqueous solutions of egg albumin irradiated with x-rays at a dose of 550,000 roentgens and different partial pressures of air. ⊗ –thiol group titer before irradiation; X-titer of thiol groups after irradiation.

Ordinary molecular oxygen apparently does not bring about any significant change in the hydrogen bonds of the protein molecule, because no aftereffect was observed in our experiments when oxygen was added (addition of oxygen after irradiation did not change the extent of oxidation of SH groups). Furthermore, if molecular oxygen exerted any such effect, protein solutions exposed to the air would have to be in a denatured state. Evidently the principal effect resulting in any such redistribution of hydrogen bonds must be exerted by oxygen molecules excited by radiation (O_2^*) or by the molecular oxygen ions O_2^-, although not in connection with any transfer of electrons along the protein molecule but rather because of a direct effect exerted on weak bonds of the protein molecule. This assumption is close to the idea advanced by R. Platzman and J. Franck [14] to the effect that the sudden appearance of a charge during the radiation ionization of the protein molecule results in a polarization wave in this molecule (reorientation of dipoles, etc.), which is accompanied by the breakage of 12-14 hydrogen bonds. The difference in the redistribution of hydrogen bonds brought about by O_2^- and O_2^* is only quantitative. The electronic structure of the oxygen molecule, which in the ground state can be characterized by the expression $KK(Z\sigma)^2(y\sigma)^2(\chi\sigma)^2(\omega\pi)^4(\nu\pi)^2$ [15], is changed in the excited state at an energy of 5-6 ev to the structure $KK(Z\sigma)^2(y\sigma)^2(\chi\sigma)^2(\omega\pi)^3(\nu\pi)^3$, where one of the $\omega\pi$ electrons rises to the $\nu\pi$ level (the ionization potential of O_2=12 ev) [16], while the energy of addition of an electron to O_2, resulting in the formation of O_2^-, may be lower than 2 ev [17]. Both the excited molecule O_2^* and O_2^- exhibit pronounced paramagnetic characteristics [18], which must be of considerable significance for the polarization effect exerted by them on the hydrogen bonds of the protein molecule.

L. Kh. Eidus and co-workers [19] explain the oxygen effect by an interaction between ordinary molecular oxygen and the excited protein molecule, having observed a pronounced aftereffect upon addition of oxygen to myosin irradiated under anaerobic conditions. In our experiments, as has been mentioned, no aftereffect was observed, so that contrary to the views expressed by Eidus, we assume that principal importance must be attached to the action of excited or ionic forms of molecular oxygen, which bring about changes in the natural structure of protein molecules.

On must note that oxygen molecules exert a similar action on nucleic acids, the natural structure of which is determined to a considerable extent by the network of hydrogen bonds. Changes in the structure of proteins and nucleic acids and also in the state of their functional groups may form the basis for a number of manifestations of the oxygen effect in the living cell.

The concepts outlined above are limited to a certain extent by the circumstance that the solutions contain a small quantity of molecular oxygen comprising approximately 1 molecule of O_2 per molecule of protein, if one starts from the solubility of oxygen in pure water. In solutions of proteins or dried preparations absorption of larger quantities of oxygen is possible because of occlusion, solubilization, etc., particularly if the molecule of oxygen is in an excited state. Furthermore, even the action of one O_2^* molecule or one O_2^- ion may bring about

scission or a regrouping of 12-14 hydrogen bonds, which is comparable with the usual extent of the scission in transformations accompanying denaturation. The study of these problems should form the subject of subsequent research.

SUMMARY

The oxidation of thiol groups has been investigated on solutions of cysteine and egg albumin and also on dry protein preparations following irradiation with x-rays or gamma rays in vacuum, in a nitrogen atmosphere, and in air (at atmospheric pressure and different partial pressures). A number of the experiments has been carried out by irradiating in the presence of denaturing substances, namely guanidine, HCl, and others.

It has been demonstrated that reinforcement of the oxidation of SH groups in the air (the oxygen effect) in cysteine solutions can be qualitatively interpreted by assuming an indirect radiation effect in such a manner that the magnitude of the oxygen effect depends on the relation between the substrate concentration and the applied radiation dose; the oxygen effect diminishes when the ratio expressing this relation drops. In this sense, the magnitude of the oxygen effect is only relative.

In protein solutions the magnitude of the oxygen effect depends to a considerable extent on the hydrogen bonds formed by SH groups. In cases of irradiation in vacuum, in a nitrogen atmosphere, or at an air pressure below 100 mm Hg, the SH groups of protein are not oxidized at all as a result of irradiation at doses up to 600,000 roentgens, following irradiation with which complete oxidation of SH groups is obtained in air. If irradiation in vacuum is carried out in guanidine solutions or irradiation takes place in air (at pressures higher than 350 mm Hg), the oxidation of SH groups proceeds normally, just as in solutions of simple thiol substances, which apparently is a result of the breaking of H-bonds in the protein molecule. Addition of air after irradiation of the protein solution does not change the SH-group titer (i. e., an aftereffect is absent).

An oxygen effect associated with the direct action of radiation apparently cannot be explained by the formation of free peroxide radicals, because the amount of oxidized SH groups exceeds by two orders of magnitude the total number of radicals that has been measured by the electronic paramagnetic resonance method. The concept is advanced that the oxygen effect associated with the direct action of radiation can be explained by the action of irradiated oxygen (present in the O_2^* or O_2^- form) on the breaking and rearrangement of a number of hydrogen bonds in the protein molecule.

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BINDING OF RIVANOL WITH SERUM PROTEINS

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The use of Rivanol (2-ethoxy-6,9-diaminoacridine lactate) as a precipitant in the fractionation of serum proteins, and in particular for precipitation of γ-globulin, was first practiced by Horejsi and Smetana [1, 2], in 1954.

It was later found that Rivanol could be used for the fractionation of tissue proteins [3], for the isolation of the complex of immunologically active fractions of serum protein [4], and for the preparation of ceruloplasmin [5] and siderophilin [6]. The use of Rivanol in protein fractionation procedures renders it most desirable to investigate the conditions under which it reacts with individual protein fractions and with multicomponent protein systems. Very little work has been published on reactions of proteins with organic cations of the acridine series [7], and with Rivanol, in particular; the above-cited papers [1-6] were, in general, devoted to problems of preparation only, and the experiments covered only a narrow range of pH values and Rivanol concentrations.

The object of the present research was to study the reactions of serum albumin and γ-globulin and of whole human blood serum with aqueous solutions of Rivanol, over a wide range of concentrations and of pH. The choice of these two variables was dictated by the importance of their role in the process of fractionation of protein mixtures with Rivanol.

A very important factor in the study of the reaction of combination of Rivanol with proteins is the selection, on a rational basis, of the optimum relative proportions of precipitant solution to protein solution. The considerable dilution of the protein solutions obtained by the procedure of Horejsi and Smetana is a hindrance to the subsequent step of lyophilization of the solutions, and interferes with the practical application of the method to preparation of individual protein fractions. Our researches showed that the degree of precipitation and the fractional composition of the protein precipitates are determined by the pH of the medium, and by the relative molar proportions of protein and precipitant taken. This finding shows that it is possible to apply a wide range of relative proportions by volume of protein and precipitant solutions.

It is more convenient, for practical purposes, to use concentrated Rivanol solutions, thus permitting of a considerable reduction in the volume of solutions to be lyophilized. We therefore paid special attention to the determination of the optimum proportions by volume of the solutions of reacting substances, in studying the conditions of reaction of whole serum and of its individual protein fractions with Rivanol. The basic experiments described in this paper involved the use of equal volumes of protein and precipitant solutions.

EXPERIMENTAL

Reaction of Rivanol with Albumin

For the study of the reaction of human serum albumin with Rivanol we took a 4% solution of protein in 0.15 M NaCl. The purity of the albumin, which was essential for the interpretation of subsequently obtained analytical data, was checked by electrophoresis. The protein content of the solutions, before and after treatment with Rivanol, was determined by the Biuret method. The final molar concentration of Rivanol ranged from 1.5×10^{-3} to 60×10^{-3} M, and the pH from 4.5 to 10.

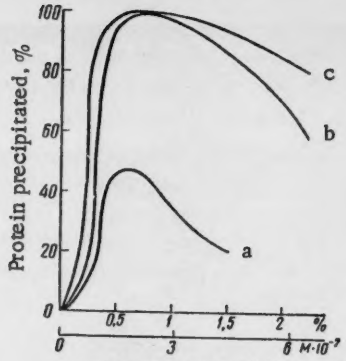

Fig. 1. Dependence of degree of precipitation of serum albumin on the concentration of Rivanol at various pH values of the solution. a) pH 6.0; 6) pH 6.5; c) pH 7.5.

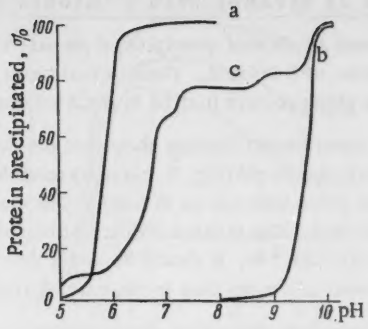

Fig. 2. Dependence of degree of precipitation of serum proteins on the pH of the solution. a) Serum albumin; b) γ-globulin ; c) whole serum.

The results of these experiments are presented in Figs. 1 and 2 (curve a), which show that the degree of precipitation of albumin rises with increasing pH from 5.2 to 7.0, and that precipitation is maximum at Rivanol concentrations of 1.16×10^{-2} M to 2.35×10^{-2} M. The position of the maximum shifts towards higher Rivanol concentrations as the pH of the solutions is increased. As the Rivanol concentrations exceed the optimum value, partial redissolution of the protein-Rivanol complex (PRC) takes place. In these experiments the pH of the solutions were kept constant (with an accuracy of ±0.05 units of pH), and it is evident that the solvent effect must have depended on the specific conditions of reaction of protein with precipitant, but not on change in the pH of the medium.

It is of interest that appreciable precipitation of protein took place at the relatively low concentration of 2.5×10^{-3} M Rivanol. With further increase in the Rivanol concentration it combines quantitatively with protein, and the resulting PRC precipitates out. Completeness of precipitation of PRC was assessed by determining free protein and Rivanol in the solution. The dye concentration was derived from measurements of the optical density at 415 mμ, which is the absorption maximum of Rivanol in the visible region of the spectrum.

The results of the spectrophotometric determination of Rivanol content are represented in Fig. 3. The curves consist of two sections, the first corresponding with full binding of dye, when the optical density is close to zero, and is unchanged by further addition of Rivanol, while the second section corresponds with presence of excess of Rivanol, the rise in optical density being proportional to the increase in concentration of free Rivanol in the solution. The small residual optical density observed in the region of total binding of Rivanol may be ascribed to partial dissolution or dissociation of the protein-Rivanol complex.

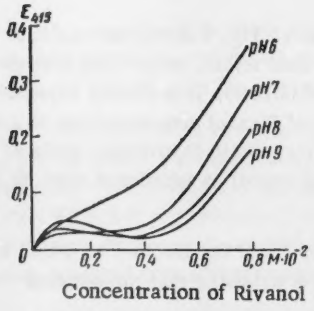

Fig. 3. Binding of Rivanol by serum albumin at different pH values.

It is noteworthy that, with increasing pH of the systems, the point of transition of the first to the second section of the curves is shifted towards higher concentrations of Rivanol. With further increase in the Rivanol concentration a second inflexion in the transmittancy curve is observed; this is due to change in the turbidity of the solutions following redissolution of precipitated PRC.

It is possible, from the curves of Figs. 1 and 3, to calculate the amount of Rivanol bound by albumin at a dye concentration at which protein is completely precipitated. Such calculations show that 1 mole of albumin combines with from 38 (pH 7) to 42 (pH 9) moles of Rivanol. With further increase in the concentration of Rivanol the number of molecules bound per molecule of protein continues to rise; a precise assessment of this number could, however, only be achieved, under our experimental conditions, within a narrow range of concentrations, corresponding with the zone of maximum precipitation.

Reaction of Rivanol with γ-Globulin *

We used an alcohol-precipitated preparation of γ-globulin (γ-globulin content, 94-96%) in our experiments on its reaction with Rivanol. These experiments were performed at pH values above and below the isoelectric point of the given protein in 0.15 M NaCl solution.

Our experimental findings show that the dependence of degree of precipitation of γ-globulin on Rivanol concentration and on pH (Fig. 2, curve b) resembles that found for albumin. In this case, however, the redissolution effect given with excess Rivanol is less clearly expressed. Precipitation of γ-globulin took place between pH 9.15 and 10.8. The Rivanol concentration for maximum precipitation at pH 10 lies within the limits 1.15×10^{-2} M to 4.5×10^{-2} M. It should be noted that precipitation of γ-globulin takes place at pH values more removed from the isoelectric point than in the case of albumin.

It follows from these data that combination of Rivanol with serum proteins is not specific to any individual fraction, but follows the general rules of reaction of organic cations with proteins. It is of interest that the reaction takes place at pH little removed from the isoelectric point (as compared with other organic cations), i. e., when the protein molecule has only a relatively small negative charge [8]. The number of molecules of Rivanol bound by γ-globulin in the zone of maximum precipitation (at pH 10) amounted to 35-42 per molecule of protein.

Reaction of Whole Serum with Aqueous Solutions of Rivanol

Having investigated the conditions under which individual protein fractions react with aqueous solutions of Rivanol, we then proceeded to the study of multicomponent protein systems, as exemplified by human blood serum. In most of the experiments we mixed equal volumes of serum and of Rivanol solution.

The protein concentrations of the sera varied within the limits 7 to 8%. In some experiments the serum protein was lowered to 4% by dilution with 0.15 M NaCl solution. Fig. 2, curve c, shows the dependence of degree of precipitation of protein on pH (at a final Rivanol concentration of 2.2×10^{-2} M). This curve shows the presence of four independent precipitation zones. The first of these, lying in the interval pH 5.5-7.2, corresponds with maximum precipitation of serum proteins. Within it up to 65% of the total protein present was precipitated. It follows, from the amount of protein precipitated, and from the pH range, that this section of the curve corresponds with quantitative precipitation of the serum albumin fraction. However, electrophoretic analysis of the sediments obtained by centrifugation of the suspensions showed that over the given pH range part of the α_1-, α_2-, β_1-, and β_2-globulins were precipitated together with the albumins. The multicomponent nature of the precipitate is probably the reason for the shift of the precipitation curve towards higher alkalinity, as compared with that for albumin.

At higher pH values the precipitate consists mostly of β_1-globulins, together with residual albumin and α_1-, α_2-, and β_2-globulins not fully precipitated at pH lower than 7.2. At this stage of the process, albumin, α_1-, α_2-, and β_2-globulins have been quantitatively precipitated, together with 70-80% of the β_1-globulin. The remaining globulin fractions are precipitated at pH higher than 9.5, using optimum relative proportions of protein and precipitant solutions. This stage of the process can, however, be achieved only when the amounts of Rivanol and protein present in the systems bear a definite relation to each other. Redissolution of the PRC precipitate takes place when either protein or Rivanol is present in excess (Figs. 4 and 5).

This effect is shown most clearly by the results of agar block electrophoresis. Fig. 4 shows the results of the electrophoretic separation of a series of fractions obtained by precipitation from serum, using rising concentrations of Rivanol (all at pH 8.4, taking equal volumes of protein and Rivanol solutions). It is clearly evident from the figure that a number of fractions undergo coprecipitation over a range of Rivanol concentrations of 1.16×10^{-2} to 2.34×10^{-2} M; over the range $2.34\text{-}2.92\times10^{-2}$ M only β_1- and r-globulins remain in solution, while at still higher concentrations part of the precipitated proteins undergo redissolution, and appear as additional spots on the electrophoretograms.

It is noteworthy that solutions of the precipitated proteins retain the properties of the fraction to which they belong, and show the same electrophoretic mobilities as they had initially; this is evidence that the proteins

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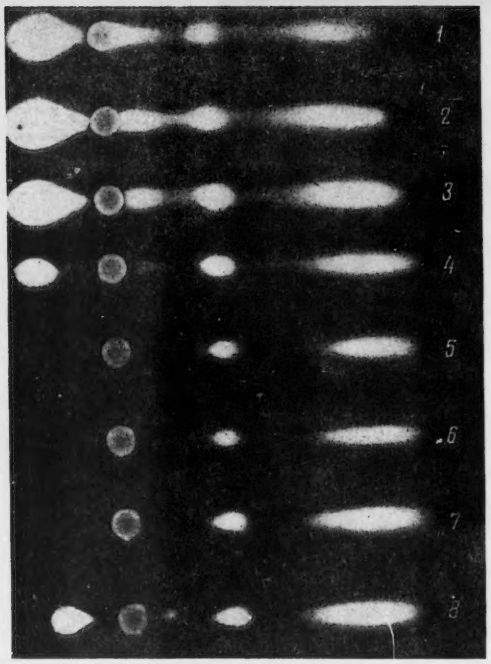

Fig. 4. Agar slab electrophoresis of specimens of serum after treatment with aqueous solutions of Rivanol at pH 8.4±0.05. 1) Initial serum, diluted 1 : 1. Final Rivanol concentration: 2) 6.58×10^{-3} M; 3) 1.16×10^{-2} M; 4) 1.75×10^{-2} M; 5) 2.34×10^{-2} M; 6) 2.92×10^{-2} M; 7) 4.39×10^{-2} M; 8) 5.85×10^{-2} M.

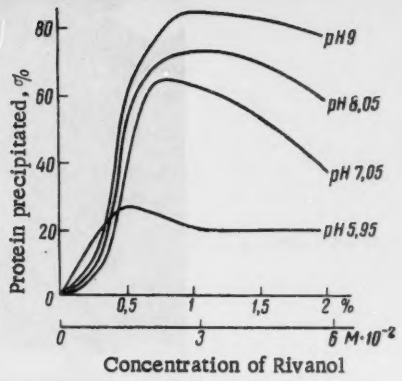

Fig. 5. Dependence of degree of precipitation of whole serum proteins on the Rivanol concentration, at different pH values of the medium.

retain their native structure when precipitated with Rivanol, and that the process is fully reversible. The redissolution effect noted by us may be ascribed to supercharging of the complexes, by adsorption on them of positively charged Rivanol ions.

Fig. 6 shows electrophoretograms of specimens of serum, precipitated using equivalent proportions of protein and precipitant in the solutions, at different ph values (samples 2-5), and with excess of protein in the solutions (samples 7-10); the final concentration of Rivanol in the systems was 2.34×10^{-2} M.

If precipitation is performed taking the optimum proportions of protein to precipitant (0.2-0.22 g Rivanol per g of protein), rising the alkalinity of the medium leads to precipitation of the entire complex of serum proteins, in the zone pH> 9.5. Under the same conditions, but in the presence of excess of protein, the precipitation process proceeds in two stages. Precipitation of albumin, α-globulin, and part of the β-globulins are precipitated over the pH interval 8.2-9.0, while at pH> 9.0 part of the previously precipitated fractions undergo redissolution; this effect appears to be associated with raising of the negative charge on the protein molecules, which cannot be compensated because of the insufficiency of positively charged Rivanol ions.

SUMMARY

A study of the reactions of serum protein with aqueous solutions of Rivanolhas shown that the degree of precipitation of the individual protein fractions is determined by three factors-the pH of the medium, the concentration of Rivanol, and the isoelectric point of the given protein.

Precipitation of albumin begins at pH 5.2, with a Rivanol concentration of from 0.16×10^{-2} to 2.35×10^{-2} M; at pH 7.0 albumin is quantitatively precipitated. Within the zone of maximum precipitation each mole of albumin combines with 38 moles of Rivanol at pH 7.0, and with 42 moles at pH 9.0. Precipitation of γ-globulins commences at pH 9.15; full precipitation is achieved at pH> 9.8, over a range of concentrations of Rivanol of $1.15\text{-}4.5 \times 10^{-2}$ M. Each mole of protein is combined with from 35 to 42 moles of Rivanol; this explains why the precipitation maximum is spread over a wider range of concentrations.

With whole serum, the zone of precipitation lies within the pH interval from 6.0-6.2 to 9.8. With the normal relative proportions of the individual protein constituents of serum protein each gram binds from 0.2 to 0.22 g of Rivanol. Formation of insoluble protein-Rivanol complexes is a reversible process. The precipitates given with

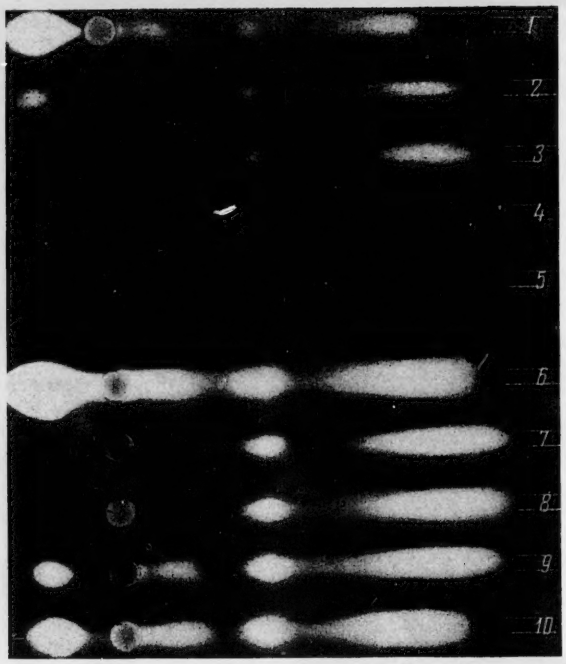

Fig. 6. Agar slab electrophoresis of specimens of whole and of diluted serum, precipitated with Rivanol at a final concentration of 2.34×10^{-2} M, and at various pH values. 1) Diluted serum, protein concentration 4.18% (1 : 1 dilution); 2-5) the same, at pH 7.5, 8.5, 9.5, and 10.2, respectively; 6) serum of protein concentration 8.36%; 7-10) the same, at pH 7.5, 8.5, 9.5, and 10, respectively.

the protein fractions (albumin, α_1-, α_2-, β_1-, and β_2-globulins) undergo redissolution in the presence of excess of Rivanol or of protein.

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OXIDATION OF VITAMIN A ALDEHYDE TO VITAMIN A ACID CATALYZED BY ALDEHYDE OXIDASE

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Vitamin A aldehyde is formed in the organism from carotene [1-3], and by the enzymic oxidation of Vitamin A [4, 5]. It can be reversibly reduced in the organism to Vitamin A alcohol [1], and it can be oxidized, either at the double bond by fatty acid peroxides, to give epoxy derivatives [6], or, as is shown in the present paper, at the aldehyde group, to give Vitamin A acid. This reaction has not, however, yet been reported in the literature, and the object of this research was to establish the conditions under which it proceeds. The reaction of formation of Vitamin A acid appears to be irreversible, since administration of the acid to Vitamin A deficient rats did not lead to accumulation of Vitamin A or its aldehyde, and to prevention or cure of xerophthalmia [7-9].

METHOD

Materials Used

Vitamin A aldehyde was prepared by oxidizing Vitamin A alcohol in petroleum ether with active manganese dioxide [10]. Vitamin A alcohol was prepared from crystalline Vitamin A acetate. Active manganese dioxide was prepared by treating manganese sulfate with $KMnO_4$ in the presence of H_2SO_4 [11]. Vitamin A aldehyde was purified chromatographically on an alumina column of a water content of 8%, and was recrystallized from petroleum ether: it could be stored for many months over CO_2 snow, without deterioration. The product was 90-95% pure; its concentration in alcohol solution was derived from its extinction $E_{1cm}^{1\%}=1400$, at 385.5 mμ.

Aldehyde oxidase. This enzyme was prepared from hog liver by Mahler's method [12], with the following modifications. Before subjecting the liver homogenate to thermal treatment we added 0.2% Na ascorbate, prepared immediately before addition by neutralizing ascorbic acid with $NaHCO_3$, to pH 7.1.

The activity of the aldehyde oxidase was assayed using acetaldehyde as substrate, by Gordon's method [13]. Protein was determined by Lowry's method [14]. We usually used enzyme preparations of the second stage of purification, when they contained 1.7 Gordon units per mg of protein, or per 0.03 ml of basic solution. In some of the experiments the enzyme was further purified according to Mahler's procedure.

Addition of ascorbate to the liver homogenate doubled the yield of enzyme obtained after the second stage of purification; the activity per mg of protein rose 1.5-fold, and the yield of protein 1.3-fold. The yields of enzyme fell drastically when autolyzed homogenates (48 hr at 5°) were taken, but when ascorbate had been added the yields were ten times greater than without its addition. The stabilizing effect of ascorbate may be ascribed to prevention of oxidation of SH groups during thermal treatment.

p-Chloromercuribenzoate. Preparation of an aqueous solution of p-chloromercuribenzoate, and spectrophotometric determination of its concentration, were effected by Boyer's methods [15].

Anionotropic aluminum oxide was prepared from chromatographic alumina by treatment with five volumes of 50% aqueous acetic acid for 10 min., with stirring. The product was washed free of acid on a Buchner funnel, until the washings had a pH of 3.5-4.0, and was stored under methanol.

EXPERIMENTAL METHOD

The aldehyde oxidase preparation was diluted tenfold in 0.1M phosphate–citrate buffer, pH 7.0, and to 0.9 ml of this solution in a centrifuge tube we added a solution of Vitamin A aldehyde in 0.1 ml of alcohol, to a final concentration of 10^{-4}M. The reaction was allowed to proceed for 15 min at 37°, and was stopped by addition of 6 ml of methanol. The concentrations of Vitamin A aldehyde and of enzyme, the reaction time, and the pH were varied, in accordance with the requirements of the given experiment. The systems were centrifuged (6000 rpm for 4 min), and the absorption spectra of the extracts were measured at 280-400 mμ, readings being taken in all cases at 335, 353, and 385.5 mμ.

For the study of the reaction products we repeated the absorption measurements after conversion of Vitamin A aldehyde hydrate into Vitamin A aldehyde by adding 1-2 drops of glacial acetic acid to 3.5 ml of the alcoholic solution, and mixing. Preliminary tests had shown that either glacial acetic acid or 50% acetic acid were the most effective for achieving this conversion, whereas 25% acetic acid, HCl, or H_2SO_4 were ineffective.

Spectrophotometric determination of the reaction products. Oxidation of Vitamin A aldehyde leads to formation of Vitamin A aldehyde hydrate and Vitamin A acid. A solution of these substances in methanol gives the composite absorption curve 1 of Fig. 1. The position of the maximum of curve 1 is displaced considerably from that of the initial aldehyde (385.5 mμ).

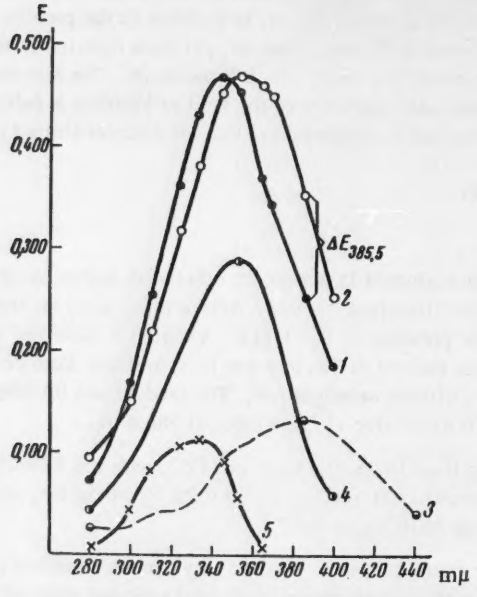

Fig. 1. Absorption curves of solutions of Vitamin A acid, aldehyde, and aldehyde hydrate in methanol. 1) Solution of Vitamin A acid, aldehyde, and aldehyde hydrate; 2) solution of Vitamin A aldehyde and acid. Component solutes of the mixture of curve 1 (calculated); 3) vitamin A aldehyde; 4) vitamin A acid; 5) vitamin A aldehyde hydrate.

After conversion of the hydrate to Vitamin A aldehyde by the action of acetic acid, the solution gave absorption curve 2 of Fig. 1. Comparison with curve 1 shows that the increase in concentration of Vitamin A aldehyde is reflected by a rise in extinction at 385.5 mμ, and by a fall in extinction at 335 mμ. The difference, shown in Fig. 1 as $\Delta E_{385.5}$, corresponds with the concentration of aldehyde hydrate.

If we designate the concentrations of Vitamin A acid and aldehyde as $E^1_{353} = E^1_{385.5}$, respectively, the following expressions can be derived:

$$E_{353}=E^1_{353}+K_1 \cdot E^1_{385.5}; \quad \text{(I)}$$

$$E_{385.5}=E^1_{385.5}+K_2 \cdot E^1_{353}; \quad \text{(II)}$$

where $K_1=0.79$, being the ratio of extinction for Vitamin A aldehyde, $E_{353}/E_{385.5}$; $K_2=0.426$, being the ratio of extinctions for Vitamin A acid, $E_{385.5}/E_{353}$. Substituting the value of $E^1_{385.5}$ from equation (II) into equation (I) gives a formula for the calculation of extinction of Vitamin A acid:

$$E^K_{353}=E^1_{353}=1.506(E_{353}-0.79E_{385.5}). \quad \text{(III)}$$

For determining the concentration of Vitamin A acid we used the extinction coefficient $E^{1\%}_1$cm=1510, at a wavelength of 353 mμ [16]. Substituting the value of E^1_{353} from equation (I) into equation (II) gives a formula for the calculation of the extinction of Vitamin A aldehyde, including the aldehyde formed from the hydrate:

$$E^1_{385.5}=1.506(E_{385.5}-0.426E_{353}). \quad \text{(IV)}$$

The amount of Vitamin A aldehyde present initially, before conversion of the hydrate form, is derived by subtracting the extinction due to Vitamin A aldehyde hydrate, $\Delta E_{385.5}$, from that due to aldehyde, E^1_{385}. The formula giving extinction due to Vitamin A aldehyde in the reaction mixture then has the form:

$$E^a_{385.5}=1.506(E_{385.5}-0.426E_{353})-\Delta E_{385.5}. \quad \text{(V)}$$

The absorption curves of Vitamin A aldehyde and acid, based on extinction values calculated from formulae (III) and (V), are given in Fig. 1 (curves 3 and 4, respectively). Curve 5, representing absorption due to aldehyde hydrate, was derived from the differences between curve 1 and the sum of the values of curves 3 and 4. The resulting absorption curve shows a maximum at 335 mμ, which coincides with the published value for Vitamin A aldehyde hydrate [17].

Chromatographic analysis of the reaction products. Chromatographic separation of methanolic solutions of the products of oxidation of Vitamin A aldehyde was effected on an anionotropic aluminum oxide column. Vitamin A aldehyde hydrate and acid differ from Vitamin A aldehyde, alcohol, and ester in being adsorbed on the column; after washing with methanol, the adsorbed substances are eluted from the column by passing 2% methanolic NH_3, when Vitamin A acid emerges first, followed by Vitamin A aldehyde hydrate. The fractions were further purified by vacuum evaporation, followed by repeated chromatographic separation of the residues.

EXPERIMENTAL RESULTS

Specificity and Singularities of Enzymic Oxidation of Vitamin A Aldehyde

As has been mentioned, Vitamin A aldehyde may undergo oxidation both at the double bond and at the aldehyde group. In aqueous colloidal solution, Vitamin A aldehyde is rapidly oxidized at the double bond, to an extent of 50% in 30 min. Spectrophotometry of the methanolic solutions showed that, as a result, absorption fell at the Vitamin A aldehyde maximum at 385.5 mμ, and rose in the region of wavelengths shorter than 280 mμ (epoxides and products of further oxidation).

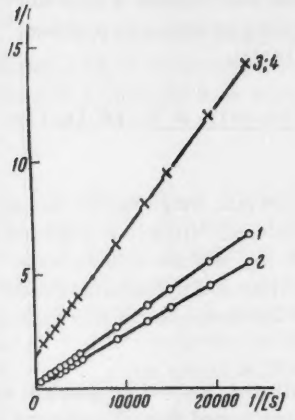

Fig. 2. Relation between the reciprocals of the rate constants of the reaction and of the substrate concentration. 1) Oxidation of Vitamin A aldehyde to acid, promoted by aldehyde oxidase, prepared according to Mahler; 2) the same, using aldehyde oxidase prepared by our modified procedure; 3) and 4) conversion of Vitamin A aldehyde into its hydrate, in the presence of aldehyde oxidase prepared by the above two procedures, respectively.

In aqueous solution, and in the presence of aldehyde oxidase, Vitamin A aldehyde is not attacked at the double bond, even after 2 hr of reaction, as is shown by the absorption measurements at 280 mμ. Spectrophotometric and chromatographic analysis on an alumina column showed that Vitamin A aldehyde hydrate was being rapidly formed. The enzymic nature of the reaction of oxidation of Vitamin A aldehyde to acid was established by the experiments involving specific inhibition of aldehyde oxidase by p-chloromercuribenzoate, and by inactivation of the enzyme by heat or by appropriate adjustment of the pH of the medium. A study was made of the enzymic reaction of oxidation of Vitamin A aldehyde, involving the following: determination of the Michaelis constant, rate of reaction, as compared with that of acetaldehyde, pH dependence, dependence on concentration of aldehyde and Vitamin A aldehyde hydrate, and examination of the rate of accumulation of products.

The Michaelis constant was derived from a graph relating the reciprocals of the reaction velocity and the substrate concentration [18]. The concentrations of Vitamin A aldehyde were varied from 0.42 to 13.4×10^{-4}M, and the reaction rate was derived from measurements of the amounts of Vitamin A acid formed. The yields of reaction products were measured spectrophotometrically. The results are presented in Fig. 2. The Michaelis constant for hog-liver aldehyde oxidase prepared according to Mahler amounted to 1.1×10^{-3}M, and for that prepared by our modified method, involving addition of ascorbate, to 1.65×10^{-3}M.

Our data for the rate of formation of Vitamin A aldehyde hydrate are also represented in Fig. 2. The Michaelis constant for aldehyde oxidase prepared by both methods was the same in this case, viz., 3.7×10^{-4}M. It thus appears that aldehyde oxidase prepared by our modification has a higher activity than that prepared by the original method, at the stage of oxidation of Vitamin A aldehyde hydrate to Vitamin A acid.

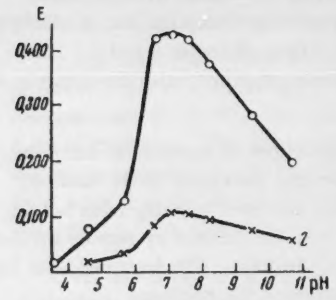

Fig. 3. Dependence of rate of oxidation of Vitamin A aldehyde on pH. 1) Vitamin A aldehyde; 2) Vitamin A aldehyde hydrate.

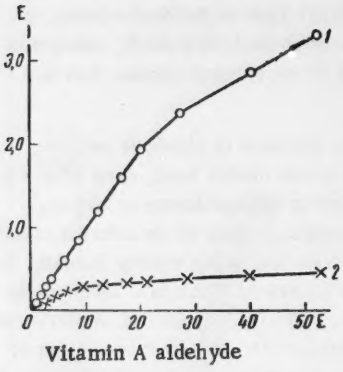

Fig. 4. Dependence of rate of oxidation of Vitamin A aldehyde on its concentration. 1) Vitamin A aldehyde; 2) Vitamin A aldehyde hydrate.

The rate of oxidation of Vitamin A aldehyde was compared with that of acetaldehyde, at optimum concentrations of the substrates, from the rate of decoloration of methylene blue, by Gordon's method [13]; it amounted to 42%. The dependence of the reaction rate on pH was investigated by the above-described spectrophotometric method; the results are represented in Fig. 3.

The pH optimum of the reaction was at 7.2; at pH 8 the activity was 91% of the maximum, at pH 6 and 9, 37 and 67.5%, respectively, and at pH 5 and10, 21.5 and 56%, respectively. The higher activity of the enzyme in the alkaline region of pH than in acid solutions may be due to the more rapid formation of vitamin A aldehyde hydrate. The enzymic reaction of oxidation of acetaldehyde shows a similar form of pH dependence: the optimum is at pH 7.5, and the activities at pH 5 and 10 are respectively 24 and 42% of the maximum.

The dependence of the rate of oxidation on Vitamin A aldehyde concentration, and the rate of accumulation of reaction products were also investigated spectrophotometrically. It appears from the results represented in Figs. 4 and 5 that the participation of Vitamin A aldehyde hydrate is confined to the relatively small amounts formed in the initial stages of the reaction. The reaction comes to an end when all of the Vitamin A aldehyde has been transformed into Vitamin A acid and into aldehyde hydrate. The reaction is fully inhibited by p-chloromercuribenzoate at a concentration of 10^{-4}M.

Possibility of Formation of Vitamin A Acid in the Organism

For these experiments we took white rats, weighing 180-250 g, maintained on the ordinary animal-house diet. Vitamin A aldehyde dissolved in oil was fed at rates of 10-60 mg, and the animals were killed by decapitation 24 hr later. The liver and the jejunum (freed of its contents) were washed with water, and immersed in five volumes of methanol, for fixation.

The tissues were then homogenized in methanol, the homogenate was centrifuged, and the supernatant was cooled at 0° for 2 hr, and again centrifuged at 0°. The clear methanolic extract was passed through a chromatography column packed with aluminum oxide, and the column was washed with methanol, to remove Vitamin A and its aldehyde, after which Vitamin A acid was eluted by passing 2% methanolic NH_3. The eluate was concentrated in vacuo, and the residue was again chromatographed. Vitamin A, Vitamin A aldehyde, and Vitamin A acid were determined spectrophotometrically.

The liver contained Vitamin A only, while the jejunum also contained Vitamin A acid. It is evident from the table that the ratio of Vitamin A acid to Vitamin A rose as the Vitamin A content of the jejunum increased.

The results shown in this table also indicate that the reaction of oxidation of Vitamin A aldehyde proceeds more slowly in the organism than does that of its reduction; this effect may be related to the lower value of the Michaelis constant in the latter case. Further investigation of this irreversible reaction in the organism should be of importance for a more complete understanding of the metabolism of Vitamin A.

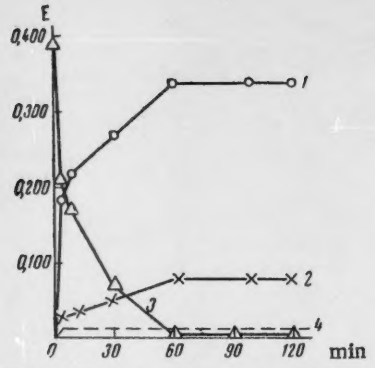

Fig. 5. Kinetics of oxidation of Vitamin A aldehyde. 1) Vitamin A acid; 2) Vitamin A aldehyde hydrate; 3) Vitamin A aldehyde; 4) control-yield of Vitamin A acid in the presence of 1×10^{-4} M p-chloromercuribenzoate.

Vitamin A Acid Content of Rat Jejunum Following Feeding with Vitamin A Aldehyde

Vitamin A aldehyde dose, mg	Vitamin A, mg-%	Vitamin A acid, mg-%	Ratio of Vitamin A acid to Vitamin A, %
0	0,02	0,00	–
10	1,2	0,08	6,7
20	4,2	0,60	14,3
60	3,0	0,27	9,0

SUMMARY

The reaction of oxidation of Vitamin A aldehyde to Vitamin A acid, promoted by aldehyde oxidase, has been established and studied. The pH optimum of the enzymic reaction is at 7.2, and the Michaelis constants of the reactions of hydration and oxidation of the aldehyde are, respectively, 3.7×10^{-4} and 1.65×10^{-3}M. The rate of oxidation of Vitamin A aldehyde is 42% of the corresponding reaction with acetaldehyde as substrate. The reaction is shown to proceed in the intact mammalian organism.

It has been shown that the yield and the specific activity of hog-liver aldehyde oxidase can be raised by adding 0.2% of Na ascorbate to the homogenate. Procedures for the determination of the products of oxidation of Vitamin A aldehyde have been elaborated.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

INFLUENCE OF RIBONUCLEOTIDES AND RIBONUCLEIC ACIDS ON RESPIRATION IN YEASTS*

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In recent years a direct participation of RNA in protein biosynthesis has been proved. It has also been demonstrated that RNA is the source of a number of cofactors which participate in metabolism [1,2]. In this connection it is of particular interest to study the influence of RNA and ribonucleotides on oxidative processes in cells.

A number of authors have demonstrated an inhibitory effect of RNA and products of its hydrolysis on tissue dehydrogenases [3-6]. Engelhardt [7], and subsequently other authors [8], demonstrated that ADP is the phosphate acceptor in oxidative phosphorylation and thereby activates respiration. From the results of Potter and coworkers [9,10] one may postulate a similar role for UDP and CDP, although Cooper and Lehninger [11] found that nucleotides with bases other than adenine participate in these processes only indirectly. Pavlova [12] believes that RNA can also be a phosphate acceptor during oxidative phosphorylation. Chepinoga and Kerova [13] found that RNA and DNA stimulate respiration in various tissues. The investigations of Khadzhiolov [14] established that RNA and nucleotide products of its hydrolysis activate respiration in yeasts.

In the present study we set ourselves the goal of studying in greater detail the influence of RNA and various ribonucleotides on the oxidative breakdown of some substrates by intact yeast cells.

MATERIALS AND METHODS

Fresh baker's yeast (Saccharomyces cerevisiae) from various commercial sources was used for the experiments. Immediately before an experiment the yeast was suspended in 1/60 M phosphate buffer at pH 6.3. Oxygen absorption was determined by a standard manometric method [15]. Two ml of yeast suspension was placed in the main part of the vessel. At the start of the experiment, substrate along with the compounds under investigation was tipped in from the side-arm. The central well contained 0.2 ml of 20% KOH. The gas phase was air, and the temperature was 30°. Oxygen absorption was determined for 120-210 minutes. All results were treated statistically. In view of the complex nature of the system (intact yeast), small changes will not be discussed, even when statistically significant.

* Abbreviations used: 5-AMP, adenosine-5'-Monophosphate; 2 (3)-AMP, isomeric mixture of adenosine--2'-monophosphate and adenosine-3'-monophosphate; 5-GMP, guanosine-5'-monophosphate; 2(3)-GMP, isomeric mixture of guanosine-2'-monophosphate and guanosine-3'-monophosphate; 5-UMP, uridine-5'-monophosphate; 2(3)-UMP, isomeric mixture of uridine-2'-monophosphate and uridine-3'-monophosphate; 5-CMP, cytidine-5'-monophosphate; CDP, cytidine-5'-disphosphate; CTP, cytidine-5'-triphosphate; 2(3)-CMP, isomeric mixture of cytidine-2-monophosphate and cytidine-3'-monophosphate; 5-NMP, nucleoside-5'-monophosphate; NDP, nucleoside-5'-diphosphates; NTP, nucleoside-5'-triphosphates.

Spectrophotometric measurements were carried out in a quartz spectrophotometer, Unicam model SP 500. Ribose was determined by the method of Maibaum as described by Schneider [16]; phosphate by the method of Weil-Malherbe and Green [17]; the mononucleotide composition of RNA preparations was determined, after preliminary hydrolysis with 0.5 N KOH and electrophoretic separation on paper, by the method of Markham and Smith [18]. Macherei-Nagel paper No. 214 was used with a 0.05 M citrate buffer at pH 3.2; the potential gradient was 25 volt/cm. Spots were detected by a mercury lamp with mercury vapor at low pressure (after Khanau, NN15/44). Spots were eluted with 0.01 M HCl, while guanylic acid was eluted with 0.1 M HCl. Absorption was determined spectrophotometrically at the absorption maximum, as given in the literature.

Seven different preparations of yeast RNA were used in the experiments: RNA from Merck; RNA_{II}, a preparation of RNA_I purified by the method of Smith and Markham [19]; RNA_{III}, a preparation purified by the method of Vischer and Chargaff, kindly donated to us by Professor I. B. Zbarskii; RNA_{IV}, a preparation donated by the Institute of Biology and Medicine, German Academy of Sciences; RNA_V, a preparation kindly provided by R. Rsanev; RNA_{VI}, a preparation from the factory of Organopreparations, Sofia; RNA_{VII}, the same as RNA_{VI}, but a different batch. All RNA preparations were dissolved in dilute KOH or NaOH (0.5–1.0 N) and then neutralized to pH 6.5 with 1 N HCl. KCl or NaCl solutions equivalent to the neutralized HCl were added to control tubes. Alkaline hydrolysis of RNA was usually carried out with 1 N KOH (20-30 mg/ml) for 18 hours at 37°. After hydrolysis the solutions were neutralized by the method just described. The following compounds were also used: 5-AMP, ADP, ATP, 5-UMP, UDP, UTP, 5-CMP, CDP, CTP, 5-GMP, DPN, TPN, adenosine, gaunosine, uridine and cytidine (obtained from the Pabst Laboratory); 2(3)-UMP, 2(3)-CMP, and 2(3)-GMP from Nutritional Biochemicals Co.; 2(3)-AMP, thiamine hydrochloride and riboflavin from Merck; and preparations of 2(3)-UMP and 2(3)-CMP were made by the method of Loring and co-workers [20]. If necessary, solutions of all compounds under investigation were previously neutralized to pH 6.5. Other materials and methods used during this study are described below.

RESULTS

Features of the Oxidation of Various Substrates. Details of the oxidation of various substrates (glucose, pyruvate, and acetate) were investigated in intact yeast cells. The typical course of oxygen uptake by yeast during the breakdown of glucose, pyruvate, and acetate is depicted in Fig. 1. Oxidation of glucose and pyruvate (Fig. 1) begins soon after addition of substrate, whereas oxidation of acetate goes on very slowly for the first 30 minutes, then speeds up significantly. It is worth noting that glucose is oxidized more rapidly than pyruvate. When the substrate is glucose, oxygen uptake soon stops, and respiration levels off at the endogenous rate. In experiments with pyruvate, oxygen uptake, though significantly lower, maintains its linear nature for a rather long time. Under the conditions of our experiments none of the substrates under study was fully oxidized. Thus, under identical conditions the breakdown of glucose was only 40%, pyruvate 45%, and acetate 65% (of the maximum possible amount from oxidation of substrate to CO_2 and H_2O). These results confirm the well-known fact [21] that under aerobic conditions a significant fraction of oxidizable substrates is converted either into products of high molecular weight (polysaccharides, proteins, etc.) or into various products of intermediary metabolism. Given high concentrations of substrate, yeasts appear not to oxidize it completely, suggesting that enzyme systems limiting substrate oxidation are present in the medium. It should be noted that according to the results of Eaton and Klein [22] and our results, the age of a culture affects the degree of oxidation of various substrates, but this circumstance demands that careful control experiments be carried out.

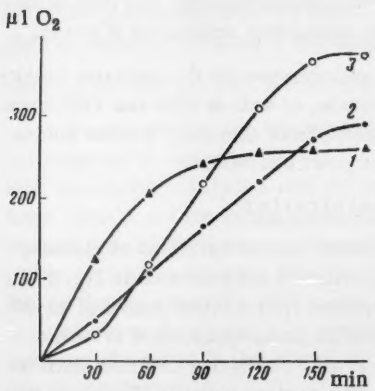

Fig. 1. Course of oxygen uptake during oxidation of various substrates. 1) Glucose, 0.1 ml of 0.05 M; 2) pyruvate, 0.1 ml of 0.1 M; 3) acetate, 0.1 ml of 0.15 M. Maximal oxygen uptake for complete oxidation of substrates to CO_2 and H_2O would be 672 μl.

Influence of RNA and Various Ribonucleotides on the Oxidation of Glucose and Acetate

We investigated the influence of RNA and its alkaline hydrolyzates, various nucleosides, 2'(3')-and 5-ribonucleotieds, and also

some derivatives (DPN and TPN) on the oxidative breakdown of glucose and acetate by yeasts. All nucleosides and nucleotides were studied at a terminal concentration of 45×10^{-4} M. The terminal concentration of RNA and its alkaline hydrolyzates was 0.18%. If the molar ratio of nucleotides in RNA is taken as 1 and its concentration is expressed on the basis of an average mononucleotide (molecular weight 302), then the terminal concentration of this "mononucleotide" will be 59×10^{-4} M. Thus, the concentration of RNA and the sum of the concentrations of the mononucleotides from its alkaline hydrolysis are of the same order of magnitude as the concentrations of the pure nucleotides. The experimental results are presented in Fig. 2.

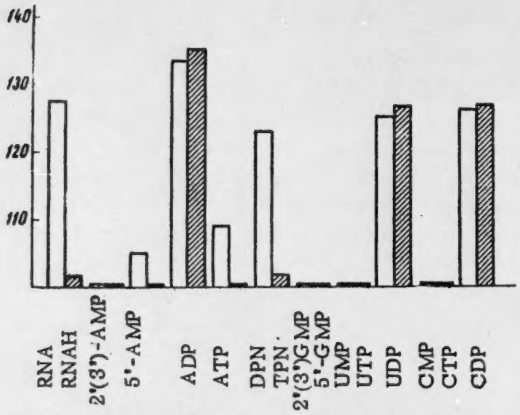

Fig. 2. Influence of RNA and various ribonucleotides on the oxidation of glucose and acetate by intact yeast. Oxidation uptake in the presence of substrate alone is taken as 100. The terminal concentration of all nucleotides studied was 45×10^{-4} M. The terminal concentration of RNA and its alkaline hydrolyzate (RNAH) was 0.18%. Unshaded columns are with glucose as a substrate (0.1 ml of 0 05 M); shaded columns are with acetate as a substrate (0.1 ml of 0.15 M).

Only some of the ribonucleotides studied—in particular, the nucleoside-5'-diphosphates ADP, UDP, and CDP—activate the oxidative breakdown of glucose and acetate and produce more complete oxidation of these substrates (Fig. 2). Pyridine nucleotides (DPN and TPN) activate only the oxidation of glucose. The other nucleotides studied—nucleoside-5'-monophosphates (5-AMP, 5-GMP, 5-UMP, and 5-CMP) and nucleoside-5'-triphosphates (ATP, UTP, and CTP)—have no pronounced effect. The activating effect of RNA and its Alkaline hydrolyzates on glucose oxidation is particularly interesting. This effect is evidently unrelated to the polymerized state of RNA. 2'(3')-Ribonucleotides (2(3)-AMP, 2(3)-GMP, 2(3)-UMP, and 2(3)-CMP) obtained from an alkaline hydrolyzate of RNA, whether tested separately or in various combinations, have no effect on yeast respiration.

The nucleosides studied (adenosine, guanosine, uridine, and cytidine and crystalline ribonuclease at a terminal concentration of 2 mg/ml also had no effect on yeast respiration.

These facts compelled us to assume that, preparations of yeast RNA (and its alkaline hydrolyzate) studied in our experiments contain some unknown component which is a cofactor for the oxidative breakdown of glucose by intact yeast cells. It should be noted that this effect cannot be attributed to the direct oxidation of the RNA, ribonucleotides, and ribonucleosides, since it was established that none of these compounds influences the endogenous respiration of yeasts.

Thus, these experiments have established that ADP, UDP, and CDP are cofactors for the oxidative breakdown of glucose and acetate by intact yeast. RNA and its alkaline hydrolyzate, as well as DPN and TPN, have an analogous effect on the oxidation of glucose without influencing the oxidation of acetate. Various nucleosides, 2'(3')-ribonucleotides, and also 5'-ribonucleotides have no effect on yeast respiration.

The Influence of RNA, ADP, DPN, and TPN on Yeast Respiration

In the following series of experiments the effect of activating compounds on the oxidation of glucose, pyruvate, and acetate was studied in more detail. The results of these experiments are presented in Fig. 3, A, B, and C. As evident from Fig. 3A, the activation of glucose oxidation appears after a latent period of 15-30 minutes. After this time the intracellular reserves of the compound in question probably decrease to such a degree that its concentration becomes the factor limiting oxidation. The rate of respiration increases until 90 minutes, after which respiration slowly levels off at the endogenous rate. The nonlinear course of glucose oxidation after addition of activator has the same character for all compounds studied. The oxidation of pyruvate (Fig. 3B) is activated soon after addition of the substances studied—well before the substrate is used up. It should be noted that RNA has no effect on the oxidation of pyruvate, while ADP, DPN, and TPN have a clearly expressed activating effect. The influence of ADP on the oxidation of acetate has a similar character (Fig. 3C). When this substrate is used, RNA, DPN, and TPN are all ineffective.

The results of these experiments show that the three groups of compounds tested influence different stages of glucose oxidation. ADP and nucleoside-5'-diphosphates activate oxygen uptake with all substrates, while DPN and TPN affect only the oxidation of glucose and pyruvate. RNA and its alkaline hydrolyzates contain a

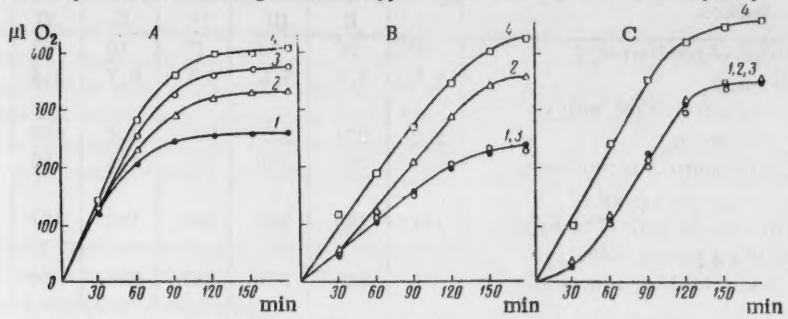

Fig. 3. Influence of RNA, ADP, and DPN on the oxidation of glucose, pyruvate, and acetate by intact yeast cells. 1) Control (substrate only); 2, 3, 4) addition of, respectively: DPN, terminal concentration 45×10^{-4} M: RNA, terminal concentration 0.18%; ADP, terminal concentration 45×10^{-4} M. A) Substrate is glucose, 0.1 ml of 0.05 M in every vessel; B) substrate, 0.1 ml of 0.1 M in every vessel; C) substrate acetate, 0.1 ml of 0.15 M in every vessel.

compound which affects only the oxidation of glucose; hence we shall henceforth call it the "factor for glucose oxidation" (FGO). On the basis of these experiments it may be concluded that FGO contained in RNA differs from all the compounds we tested related to nucleotides (ADP, DPN, and TPN), which activate the oxidation of glucose.

Probably the participation of ADP in respiration as a cofactor is related to its function as a phosphate acceptor during oxidative phosphorylation. Hence we studied the influence of sodium azide and 2, 4-dinitrophenol (DNP) on the activating effect of RNA and ADP during the oxidation of glucose. It was established in preliminary experiments that sodium azide and DNP at terminal concentration of 1×10^{-4} M have no influence on oxygen uptake by yeast; from the results of Seits and Engel'gardt [23], these concentrations block oxidative phosphorylation. It was established in our experiments that sodium azide and DNP at these concentrations eliminate the activating effect of ADP on glucose oxidation but do not interfere with the effect of RNA. A more complete analysis of the effect of sodium azide and DNP, in combination with various nucleotides, on glucose oxidation by yeast is the subject of a separate investigation [24].

Effect of Different RNA Preparations on Yeast Respiration

To test whether the observed effect of RNA (or of the supposed FGO) on glucose oxidation depends upon the presence of accidental components or whether it is connected with a definite chemical property of RNA itself, we carried out studies with different preparations of yeast RNA of different degrees of purity. As seen from Table 1, all the preparations studied have an obvious activating effect. Despite identical terminal concentrations, the different preparations activate the oxidation of glucose to an extent varying from 16 to 42%, in comparison with controls. Alkaline hydrolyzates of different RNA preparations showed the same activating effect as the corresponding RNA preparations. This shows that the difference in activity of different preparations is unrelated to the degree of polymerization of RNA. Different preparations were analyzed for the following indices: absorption in the ultraviolet (after preliminary hydrolysis), ribose content of purine nucleotides, and phosphorus content.

The phosphorus content in the preparations studied varies from 5.4 to 7.5% and is significantly lower than the theoretical maximum for an average "mononucleotide" (10.3%, Table 1). The fact that the absorption at 260 mµ, computed on the basis of phosphorus content, is very close to the value found experimentally excludes the possibility of attributing the low phosphorus content in RNA preparations to dephosphorylation. In all preparations (except for RNA_{VI}) the ribose content, as computed theoretically on the basis of the phosphorus content, is significantly lower than the value found. This implies that the preparations of yeast RNA studied contain more

TABLE 1. Characteristics of Various RNA Preparations and Their Influence on Oxidation of Glucose by Intact Yeast Cells

Indices	RNA preparations						
	I	II	III	IV	V	VI	VII
Activation of respiration, %	22	26	32	17	16	42	14
Phosphorus, %	7.2	7.5	6.6	6.8	6.7	5.4	5.8
$E^{1\%}_{260}$, computed on the basis of phosphorus	262	274	241	247	244	198	210
$E^{1\%}_{260}$, determined experimentally	261	265	271	240	242	82	196
Ribose, in μg per mg RNA, computed on the basis of phosphorus	174	183	160	164	162	131	140
Ribose, in μg per mg RNA, determined experimentally	205	233	273	233	266	50	225

purine nucleotides than pyrimidines. The absorption spectra of the different RNA preparations corresponded to those described in the literature [25]. Determination of the absorption maxima of different preparations gave the following values: I, 260 mμ; II, 258 mμ; III, 258 mμ; IV, 260 mμ; V, 259 mμ; VI, 262 mμ; VII, 259 mμ.

TABLE 2. Mononucleotide Composition of the Experimental Preparations of Yeast RNA, in Mole %

Mononucleotides	RNA preparations					
	I	II	III	IV	V	Average
AMP	27.0	27.8	27.5	27.2	29.9	27.9
GMP	30.4	28.8	29.0	30.0	28.5	29.3
UMP	21.6	22.0	22.7	23.4	20.1	21.9
CMP	21.0	21.4	20.8	19.4	21.5	20.9
Purines/ pyrimidines	1.35	1.30	1.25	1.34	1.40	1.34

As seen from Table 2, the results we obtained for mononucleotide composition of yeast RNA are in accord with those given in the literature [31,32]. Particularly noteworthy is the constancy of the purine/ pyrimidine ratio, which often does not receive attention when yeast RNA is used as a standard to determine RNA in different materials [30].

The evidence concerning characteristics of different preparations of yeast RNA shows that the RNA preparations used (apart from RNA_{VI} and RNA_{VII}) were sufficiently pure. No link was established between the activation or respiration by RNA and its mononucleotide composition, absorption in the ultraviolet resorcinol test, and phosphorus content. These results support our assumption that the activation of respiration depends upon substances in the RNA or components of its structure which are not identical with the four known mononucleotides.

Experiments to Identify the Factor for Glucose Oxidation

We conducted some preliminary experiments to identify the "factor for glucose oxidation" contained in RNA preparations. The experiments were carried out on RNA.

RNA was dissolved in water, dialyzed for a day against distilled water, and the dialyzate was dried by lyophilizing. The result was an amorphous, yellow, strongly hygroscopic powder. When dissolved in water, this powder had the same effect on oxidation of glucose by yeast as the original RNA preparation. However, the ultraviolet absorption spectrum of the dialyzate was typical for RNA. Evidently the RNA preparation was depolymerized, so that polynucleotides, entering the dialyzate along with FGO, rendered dialysis ineffective for separation of FGO from RNA. Extraction of RNA for 16 hours in a Soxhlet apparatus did not decrease its effect on glucose oxidation. The same results were obtained with extraction of RNA by ether, chloroform, benzene, and acetone.

An RNA solution in NaOH (25 mg/ml) was prepared under sterile conditions, and stored at 25° for 17 days, after which it neutralized (see section under "Methods"). It was established that prolonged storage of RNA in alkali decreases its stimulatory effect on glucose oxidation by yeast by approximately 40%.

In view of the role of thiamine and riboflavin in oxidative processes, the oxidation of glucose was investigated in the presence of these vitamins. Thiamine (terminal concentration up to 0.01 M) and riboflavin (up to 1×10^{-4} M) had no effect on yeast respiration. Since riboflavin activates respiration at higher concentrations, the RNA preparation was tested for the presence of flavins [28]. The yellow fluorescence characteristic of flavins was not detected on chromatograms. Considering the high sensitivity of the method (from our results, 0.05 μg of flavin), one must conclude that if the preparations studied contained a trace of flavin, its quantity did not exceed 0.001%.

The preliminary experiments suggest that FGO contained in RNA preparations can be dialyzed, does not dissolve in organic solvents, and is resistant to acids and alkalis. There is reason to suppose that FGO is not identical with known cofactors of oxidation.

DISCUSSION OF RESULTS

These results raise a number of questions concerning the mechanism of the ribonucleotide effect on yeast respiration. Since the experiments were carried out on intact cells, the possibility cannot be completely excluded that the observed effects result from a change in cellular permeability. However, we believe that the effect of nucleotides depends upon their penetrating into yeast cells and praticipating directly in oxidative processes. This is indicated by the following facts: sepcificity of the effect (only NDP effective), activation of the oxidation of different substrates by different cofactors; action of cofactors as catalysts, permitting more complete oxidation of the substrates studied and not only increasing the rate of oxidation, as would be observed for an ordinary increase in permeability.

In view of published results [8] on the role of ADP as a phosphate acceptor during oxidative phosphorylation, we believe that the activating effect of NDP on yeast respiration which we observed is to be explained by a direct participation of NDP in processes of oxidative phosphorylation. This is suggested by the following facts: first, NDP equally activates the oxidation of all substrates studied; secondly, this effect is specific for NDP alone, whereas NMP and NTP have no effect on respiration. Finally, the elimination of the activating effect of NDP by inhibitors of oxidative phosphorylation also supports this suggestion as to the mechanism of its participation in respiration. Our experiments proved that not only ADP, but also UDP, and CDP activate respiration. The activating effect of ADP is somewhat greater than that of UDP and CDP, which is reminiscent of the primary role of ADP as a phosphate acceptor [11]. Evidently the effect of UDP and CDP is connected with a very active nucleoside transphosphorylase in yeast cells, regenerating ADP from the ATP formed during oxidative phosphorylation. At the same time the systems UDP⇌UTP and CDP⇌CTP (and evidently GDP⇌GTP) lead to formation of high-energy phosphate and then to its reversible transfer to the system ADP⇌ATP, or else to reactions in other metabolic pathways [1; 2; 9; 10]. It is interesting to note that muscle, which has a similar nonnucleotide system (creatine⇌creatine phosphate), contains smaller amounts of all nucleotides with bases other than adenine, in comparison with other tissues [29].

The activating effect of DPN and TPN, established in our experiments, is confined to the oxidation of glucose and pyruvate alone. This compels us to believe that the concentration of DPN and TPN is a limiting factor in the conversion of pyruvate to acetate. From the evidence of Holzer and Goedde, the conversion of pyruvate to acetyle coenzyme A can occur by two routes, of which only one uses DPN and TPN as cofactors, while the other is activated only by DPN. In our experiments DPN and TPN have an identical effect, implying that they activate acetaldehyde dehydrogenase, the activity of which limits the oxidation of glucose and pyruvate [30].

The activating effect of RNA and its hydrolyzates on glucose oxidation is particularly interesting. The section of "Results" described the facts which suggest that the observed effect is not caused by RNA itself or its component nucleotides. The substance which is contained in preparations of yeast RNA and which has an activating effect of glucose oxidation – i.e., FGO – is evidently tightly bound to RNA, since all the preparations studied showed this effect. Since FGO is unchanged after hydrolysis of RNA and is unaffected by inhibitors of oxidative phosphorylation, it can hardly be identified with the polynucleotide cofactor of oxidative phosphory-

lation discovered by Pinchot [31]. The absence of an effect of RNA and its hydrolyzates on pyruvate oxidation makes the identification of FGO with DPN or TPN unlikely. Preliminary experiments to identify FGO could not relate it to known cofactors of oxidative processes. Since our RNA preparations were rather pure, and since, on the other hand, FGO appeared in all preparations studied, we believe it quite possible that FGO is one of the so-called trace components of RNA.

As for the metabolic step affected by FGO, the fact that it does not influence the oxidation of pyruvate and acetate compels us to assume that it activates the oxidative breakdown of glucose by the pentose phosphate shunt. The direct oxidation of glucose in yeast by the shunt pathway has been demonstrated by a number of authors [21; 32], but the factors influencing this oxidation are still unkonwn. On the other hand, the number of known cofactors of shunt glucose breakdown is rather small [33], suggesting that some are still unknown. Obviously, the question of the importance of FGO and its distinction from known cofactors cannot be regarded as finally settled; a detailed analysis of these results is the subject of future investigations. At present, it seems necessary to stress that conclusions about one or another biological role of RNA must be drawn only with great care.

SUMMARY

Nucleosides, and also nucleoside-2'(3')-monophosphates, nucleosides-5'-monophosphates, and nucleoside-5'-triphosphates, have no effect on glucose and acetate oxidation in yeast. Nucleoside-5'-diphosphates activate the more complete oxidation of glucose, pyruvate, and acetate, while UDP and CDP are less effective than ADP. Thus, ADP, UDP, and CDP evidently activate respiration as phosphate acceptors in oxidative phosphorylation.

Pyridine nucleotides (DPN and TPN) catalyze the more complete oxidation of glucose and pyruvate but do not affect acetate oxidation. This effect is evidently related to their role as cofactors for acetaldehyde dehydrogenase, which limits pyruvate oxidation.

Different preparations of yeast RNA and also their alkaline hydrolyzates activate glucose oxidation without affecting pyruvate and acetate oxidation. This effect is not dependent on RNA itself or its constituent ribonucleotides but probably on an unknown factor in RNA preparations; and this factor probably praticipates in the oxidative breakdown of glucose by the shunt pathway. This factor is provisionally called the "factor of glucose oxidation".

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OXIDATIVE CONVERSIONS OF LIPIDS IN IRRADIATED PLANT LEAVES

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Exposure of plants to ionizing radiation causes biochemical changes as a result of the disturbance of normal metabolic processes. The investigation of these changes is of considerable interest, for the primary biochemical changes may be detected before the structural damage to the cells becomes evident. Many disturbances of normal biochemical reactions are characterized by intensive oxidation. The disturbance of enzymic processes in an irradiated organism is accompanied by changes in the concentration of the substrates, which leads to alterations in the sequence of the enzymic processes.

Of great interest are the biochemical changes associated with the formation of toxic substances in the irradiated organism, for example peroxides of lipids [1], unsaturated fatty acids [2] and other toxic, diffusible substances [3].

We have previously shown that the activity of an enzymic system oxidizing unsaturated fatty acids – lipoxidase – in the leaves of irradiated plants of various species (wheat, barley, haricot beans, soy and peas) is increased by 30-50% during the first 24 hours after irradiation [4-6]. Investigation of the chemical changes in the free and combined lipids in haricot bean leaves after irradiation showed that the peroxide content in the free lipid fraction of irradiated leaves is considerably higher than that in unirradiated leaves [7,8].

The study of the changes in the composition of the free lipids during irradiation of plant tissues was the aim of the present research.

EXPERIMENTAL METHOD

Shoots of the Latvia variety of haricot bean and of the Wiener variety of barley, grown in a hothouse, were irradiated when 10-14 days old. Before irradiation the plant shoots were carefully dug from the soil and placed in glass tumblers containing tap water. The roots of the plants were first washed free from soil. Irradiation was carried out by means of the RUP-1 apparatus, at a dose rate of 595 r/min and a focus distance of 22 cm. The diameter of the leaf crown did not exceed 9-11 cm. The material was lyophilized 2, 4, 6 or 24 hours after irradiation.

During the investigation of the free and combined lipids in the plant leaves after irradiation, the lipids were extracted with petroleum ether at room temperature by the method of Zirm, Pongratz and Polesofsky [9], which we described in detail in our previous paper [7]. Determination of the peroxides in the free and combined lipids of the control and irradiated plants was carried out by the method of Glavind and Hartmann [10], which we also described in our previous paper [7]. Chromatographic separation of the free lipids and unsaturated fatty acids of the haricot bean leaves was undertaken by the method of Spitery [11] and Alimova [12]. Leningrad factory No. 2 paper was used, after the following preliminary treatment: strips of paper were soaked in a 10% solution of vaseline oil in benzene for 24 hours, and then dried under exhaustion until the odor of benzene had disappeared.

The fatty acids were separated by means of glacial acetic acid for 20-24 hours at room temperature. The chromatograms were dried in air and washed 2-3 times with distilled water. The unsaturated fatty acids were developed with 1% $KMnO_4$ for 5-7 minutes [12], after which the paper strips were quickly rinsed free from permanganate with water. The general background of the paper remained pale yellow, and the fatty acids were developed as contrasting yellowish-brown stains, which were not decolorized after a relatively long period of time.

Labels, in the form of pure unsaturated fatty acids, for the performance of experiments on the separation of these acids by paper chromatography, were obtained by fractionation of fatty acids after saponification of vegetable oils or animal fats by the method of Swern and Parker [13]. Fractionation of fatty acids with urea was carried out as follows: 10 g of the mixture of fatty acids after saponification of the oil was added to a hot solution of urea in methanol (13.1 g urea in 32.7 ml hot methanol with subsequent heating until the urea was completely dissolved). After the addition of the fatty acids the mixture of reagents was slowly cooled to room temperature. During this procedure fine needle crystals of the complex of unsaturated fatty acids with urea were precipitated from the solution, removed by filtration through a Buchner funner with aspiration, and washed 2-3 times with pure methanol. The filtrate was evaporated on a water bath at 50° until no further precipitate was formed. To the dish containing the precipitate was then added 2.65 g urea, dissolved in 25 ml of hot methanol, and the mixture was heated until the urea had completely dissolved, and then slowly cooled. The crystals of urea–acid complexes were again removed by filtration and washed with methanol. The filtrate was again evaporated and the precipitation of the fatty acids repeated twice more. After 3-4 precipitations the filtrate contained 95% of unsaturated fatty acids, and all the saturated fatty acids were concentrated in a complex with urea. The complex of fatty acids with urea was decomposed with HCL and the saturated fatty acids were extracted with petroleum ether from an acid solution. The methanol was removed by evaporation from the filtrate containing the unsaturated fatty acids, and the residue, consisting of a mixture of acids, was redissolved in petroleum ether. The ethereal solution was washed free from urea with water and dried under anhydrous Na_2SO_4. Subsequent fractionation of the unsaturated fatty acids was carried out by the method of fractional crystallization at low temperatures [14].

TABLE 1. Influence of Ionizing Radiation on the Peroxide Content of the "Free Lipids" Fraction in the Leaves of the Shoots (Mean values of 2-3 parallel experiments given; weight of sample of dry material ≤ 0.3 g per experiment)

Time after irradiation, hours	Conditions of experiment	Content of peroxides: mg equivalent/1 kg dry weight	Content of peroxides: %	Time after irradiation, hours	Conditions of experiment	Content of peroxides: mg equivalent/1 kg dry weight	Content of peroxides: %
	Haricot beans				Haricot beans		
1/6	Unirradiated	2,450	100.0	24	Unirradiated	2,670	100.0
	1000 r	5,970	243.7		10 000 *p*	3,477	130.2
	10 000 r	10,640	430.2				
1/4	Unirradiated	1,730	100.0		Peas		
	1000 r	2,270	145.2				
	10 000 r	3,930	226.7	1/4	Unirradiated	2,960	100.0
2	Unirradiated	1,537	100.0		10 000 *p*	7,200	243.0
	1000 r	2,649	172.2				
	10 000 r	2,846	182.7		Barley		
2	Unirradiated	3,793	100.0				
	1000 r	5,66	149.3	6	Unirradiated	16,600	100.0
	Unirradiated	3,793	100.0		10 000 *p*	83,800	505.0
2	10 000 r	4,487	118.3	2	Unirradiated	4,300	100.0
	Unirradiated	1,730	100.0		10 000 *p*	5,400	120.9
	1000 r	2,250	145.0	2	Unirradiated	0,700	100.0
4	10 000 r	4,720	261.2		10 000 *p*	0,900	125.0
24	Unirradiated	0,411	100.0	24	10 000 *p*	2,500	375.0
	1000 r	2,300	559.0				
24	Unirradiated	8,425	100.0				
	10 000 r	13,220	157.6				

EXPERIMENTAL RESULTS

After irradiation of leaves with a dose of 1000-10,000 r we observed marked changes in the lipid content. It can be seen in Table 1 that the free lipid fraction in the irradiated leaves of the shoots contained 2-6 times more peroxides than that in the unirradiated leaves. Determination of the peroxides in the "combined lipids" fraction showed that a very insignificant amount of peroxides was formed during irradiation – 0.16-0.42 mg equiv./kg dry weight – comprising 3-5% of the control value. The peroxide content in the combined lipids fraction changed during irradiation, however, in a similar manner to the change in the peroxide content in the free lipids fraction. It also follows from Table 1 that the absolute content of peroxides in the same culture is not always identical. We observed the highest content of peroxides in the leaves of 10-day old shoots during spring and summer. In autumn and winter the peroxide content in the leaves was much smaller, and moreover in both the control and the irradiated plants the probable cause of this phenomenon was the less intensive metabolism during these seasons.

We next separated the free lipids fraction of the haricot bean leaves by the paper chromatography method [11,12] before and after irradiation of the plants. As a first step a comparative evaluation was made of the various methods of extraction of the lipids and preparation of the solution, and of the different conditions of performance of the chromatographic analysis. As a result of these experiments we devised a method of chromatographic analysis of the composition of the free lipids fraction using various Soviet grades of chromatographic paper.

Extraction of the lipids from the leaves of the irradiated and unirradiated haricot bean plants was carried out as follows: A sample of leaves weighing 1 g or 2 g was ground up in a mortar, cooled with dry ice. The finely ground material was transferred to a flask containing 30-50 ml of cold petroleum ether, and lipid extraction carried out [9]. The residue was removed by filtration and the ethereal solution was evaporated to a volume of 1-2 ml in a current of CO_2 and saponified with 5% KOH at room temperature. The salts of the fatty acids, in alkaline solution, were converted to the free acids by neutralization of the solution with acetic acid; the fatty acids were extracted with petroleum ether and the ethereal extract was washed free from acetic acid with distilled water and dried over Na_2SO_4. The ether was then evaporated in a current of CO_2, and the residue, containing fatty acids, was dissolved in a 3:2 mixture of ethyl alcohol and petroleum ether. The mixture of fatty acids thus obtained from the haricot bean leaves was transferred to paper strips soaked in vaseline oil in benzene, and separated by means of the ascending movement of the solvent.

During paper chromatography, the extract of the leaves of the control, unirradiated haricot bean plant four well-defined stains (Fig. 1). When we used our solutions of pure linoleic and oleinic acids as indicators, stains of linoleic, oleinic, erucic and euconogenic acids were observed to appear on the chromatogram.

Irradiation of haricot bean leaves in a dose of 20,000 r caused considerable changes in the content of unsaturated fatty acids of the free lipids fraction. Only four hours after irradiation all the unsaturated fatty acids had almost completely disappeared from this fraction (Fig. 2.).

In order to answer the question of the nature of peroxide formation in leaves during irradiation of plants, we carried out experiments in which the lipoxidase contained in the shoots was inactivated by steaming the leaves during irradiation of plants, we carried out experiments in which the lipoxidase contained in the shoots was inactivated by steaming the leaves for 7-10 minutes. The lipoxidase activity of the steamed leaves was tested by a manometric method. As a result it was shown that the lipoxidase in this case was completely inactivated.

Experiments were conducted in which steamed haricot bean leaves of varieties Latvia and Hybrid 841 were irradiated. Unirradiated, fresh leaves from shoots acted as controls. The experiments were carried out on steamed leaves, irradiated leaves, and leaves irradiated after preliminary steaming in a dose of 10,000 r. The results obtained are shown in Table 2. Irradiation of leaves in which the lipoxidase was inactivated by steam (Table 2) did not lead to any increase in the peroxide content, whereas irradiation of the leaves of the unsteamed control plant, carried out in a parallel series, caused a sharp rise in the content of lipid peroxides.

DISCUSSION OF THE RESULTS

It has been shown by a series of investigations [15] that considerable biochemical changes take place during the action of ionizing radiation on plants. These changes affect the lipid metabolism of the plant, causing considerable oxidative conversions of the lipids. In the recent literature attention has been directed to the formation of organic peroxides and hydroperoxides in biological substrates under the influence of ionizing radiation. In radiation injuries these peroxides exhibit considerable biochemical activity and toxicity.

It follows from the results obtained that, simultaneously with an increase in the lipoxidase activity which we have previously pointed out [5-8], there is an increase in the content of lipid peroxides in the leaves of plants irradiated with comparatively small doses (1000 r). By comparison with the unirradiated leaves, the peroxide content in the free lipids fraction of the irradiated plants increased roughly 2-6 fold. Further evidence of the considerable oxidative conversions of lipids is also given by the results of experiments on the separation of the free lipids fraction by means of the paper chromatography method, showing that during the action of ionizing radiation considerable changes take place in the composition of the unsaturated fatty acids. The oxidation of the plant lipids which is observed is due to the action of enzymes in vivo. For instance, from the figures given in Table 2 it is clear that in the experiments in which the lipoxidase contained in the shoots was inactivated by steaming the leaves, no increase took place in the peroxide content, whereas irradiation of the fresh leaves led not only to activation of the lipoxidase but also to a considerable increase in the lipid peroxides.

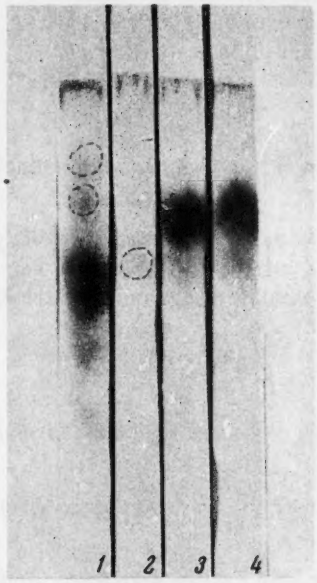

Fig. 1. Chromatogram of the free lipids fraction from unirradiated haricot bean leaves (control). 1) Extract of free lipids fraction; 2) indicator–oleinic acid; 3) indicator–linoleic acid; 4) indicator–mixture of linoleic and oleinic acids.

The results of the present investigation confirm the view that experiments conducted in vitro cannot reporduce conditions even remotely resembling those which actually exist inside the living cell. In particular, our previous investigations showed that the enzyme system oxidizing unsaturated fatty acids, namely lipoxidase, is resistant to the action of oxidation in experiments in vitro, whereas in experiments in vivo the lipoxidase activity in the leaves of irradiated plants is

TABLE 2. Influence of Ionizing Radiation on the Peroxide Content of the Free Lipids Fraction in the Leaves of Shoots Inactivated by Steam

Time after irradiation, hours	Conditions of experiment	Peroxide content	
		mg equiv. per kg dry weight	%
1/6	control	2.450	100.0
	1000 r	5.970	243.7
	steamed + 1000 r	1.140	46.5
24	control	6.485	100.0
	steamed	4.660	71.9
	steamed + 10,000 r	1.829	28.2
24	control	5.423	100.0
	steamed	4.841	89.3
	steamed + 10,000 r	4.803	79.4
24	control	106.4	100.0
	10,000 r	161.0	150.0
	steamed + 10,000 r	62.9	59.1

increased during the first 24 hours after the action of radiation [16,17]. The increase in the content of lipid peroxides in the leaves of irradiated shoots, in addition to the increase in the lipoxidase activity, demonstrate that enzymic oxidation of lipids takes place in plants under the influence of ionizing radiation. Evidently the changes in the composition of the unsaturated fatty acids in the free lipids fraction (arising under the influence of irradiation: disappearance of linoleic, oleinic and other acids within the first 24 houts after the action of

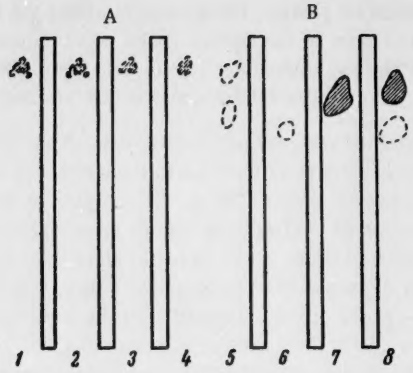

Fig. 2. Diagram showing the distribution on the chromatograms of the unsaturated fatty acids contained in the free lipids fraction from haricot bean leaves (10-day old shoots), irradiated in a dose of 20,000 r. A) 4 hours after irradiation: 1-4) extract of the free lipids fraction in volumes of 0.07; 0.09; 0.11 and 0.10 μl respectively of free lipids fraction – 0.100 μl; 6-8) indicators: oleinic acid – 0.015, linoleic acid – 0.02; mixture of linoleic and oleninic acids – 0.015 + + 0.02 μl respectively.

radiation) are also associated with the presence of a stage of activation of lipoxidase and with a simultaneous increase in the content of lipid peroxides.

From this point of view the study of the conditions of formation of peroxides of unsaturated fatty acids, the identification of lipid peroxides formed during irradiation of plants, and the investigation of their physiological properties may prove useful in the case of research unconnected with the problem of radiation damage of living organisms.

SUMMARY

The effect of ionizing radiation on the change in the composition of the free lipid fraction of various plant species, such as bean, pea and barley, was studied.

The results show an appreciable increase in the lipid peroxide content in the leaves of the shoots irradiated with relatively low doses (1000 r).

Changes in the composition of unsaturated fatty acids of the free lipid fraction were found within the first hours following exposure to ionizing radiation. Linoleic, oleic and other unsaturated acids disappear completely from this fraction.

Oxidation of plant lipids is caused by the in vivo effect of enzymes since no such increase in lipid peroxides has been noted upon irradiation of the substrates of lipid metabolism (such as linoleic acid, carotene and lipoxidase), and of leaves in which lipoxidase has been inactivated by steam.

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THE NUCLEOTIDE METABOLISM OF THE BONE MARROW IN NORMAL CONDITIONS AND AFTER IRRADIATION WITH X-RAYS

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Previous researches [1,2] have shown that the content of acid-soluble purine and pyrimidine compounds in the bone marrow increases considerably 48 hours after local x-ray irradiation in a dose of 2000 r. The autolytic formation of these compounds in consequence of the breakdown of RNA 48 hours after irradiation is also inhibited two- or threefold [2]. It has been postulated, moreover, that the reaction of phosphorolysis is present in the bone marrow, since the autolytic breakdown of the RNA of the bone marrow in an orthophosphate buffer solution was 2-3 times more intensive than in a pyrophosphate buffer solution [2].

In connection with this hypothesis, in the present research we investigated the action of bone marrow extracts on RNA and ribonucleotides in various buffer solutions. Data are also presented, showing the changes in the content of acid-soluble purine and pyrimidine compounds and the changes in the intensity of the autolytic breakdown of RNA in the bone marrow at different times after irradiation with x-rays.

EXPERIMENTAL METHOD

Irradiation. As experimental animals rabbits weighing 2.2-2.6 kg were used. One (the right) hindlimb of the animal was irradiated, the rest of the body being screened with lead 5 mm thick. The source of irradiation was the RUP tube. Conditions of irradiation: 180 kv; 15 ma; λ 0.15 A; filters 0.5 mm Cu and 0.75 mm Al; duration of exposure 22 min 24 sec; focus distance 30 cm; dose 2000 r. At different periods after irradiation the rabbits were sacrificed by decapitation and exsanguination. The bone marrow was extracted without delay and placed in the refrigerator (T 3°), that from the right and left lower limbs being kept separately. The content of acid-soluble purine and pyrimidine compounds and also the enzymic activity (ribonuclease activity and autolysis) of the bone marrow homogenates were determined.

Preparation of acid extracts of bone marrow. In order to determine the content of low-molecular purine and pyrimidine compounds (mainly ribonucleotides [2]) and for the investigation of autolysis of the bone marrow we used the method of extraction with 0.6N $HClO_4$ in the cold at 3°. To a weighed sample of 0.3 g of bone marrow we added 3.5 ml of 0.3M orthophosphate buffer solution at pH = 7.0. The bone marrow was carefully ground up and the proteins and nucleic acids precipitated with 1.2N $HClO_4$ in a volume equal to the volume of orthophosphate buffer solution added. For the determination of the content of nucleotides, $HClO_4$ was added immediately after homogenization of the bone marrow, and for investigation of the autolysis, before and after incubation. The addition of the orthophosphate buffer solution results in the more complete extraction of preformed nucleotides, since phosphate facilitates the transfer of proteins into the solution and their subsequent precipitation in the form of a more finely dispersed precipitate than is obtained by extraction without the addition of an orthophosphate buffer solution.

After separation of the residue of proteins and nucleic acids, the chloric acid filtrates were neutralized with concentrated KOH, using phenolphthalein and bromcresol purple as indicators. The precipitate of $KClO_4$ was removed by filtration. Repeated extraction of the tissue with $HClO_4$ without orthophosphate gives the same total yield of nucleotides as a singel extraction with $HClO_4$ and preliminary homogenization of the tissue in orthophosphate. In all the experiments, parallel tests were carried out.

EXPERIMENTAL RESULTS

The Action of Bone Marrow Extracts on RNA and Ribonucleotides in Various Buffer Solutions

The enzymic breakdown of RNA in various buffer solutions. In research into the determination of the ribonuclease activity of the bone marrow [3,4] it is customary to use succinic acid, boric acid and other non-phosphate buffer solutions. In connection with the investigation of the possibility of phosphorolysis of RNA in the bone marrow we carried out experiments on the enzymic decomposition of RNA in orthophosphate, pyrophosphate and tris buffer solutions.

Experiments were carried out with two different commercial preparations of yeast RNA. Composition of the tests: 20 mg RNA in 1.5 ml solution, pH 7.0 + 1.65 mg Mg^{++} acetate in 0.3 ml H_2O + 1.5 ml 0.3M buffer solution, pH 7.0 + 0.5 mg bone marrow in 0.2 ml H_2O, pH 7.0. With each buffer solution tests were carried out with and without incubation at 37° for 60 minutes. With this quantity of bone marrow per test, the rate of breakdown of RNA did not attain the maximum values at which it becomes difficult to compare the intensity of the enzymic decomposition of RNA in different conditions.

After incubation (and in the controls, without incubation) the proteins and nucleic acids were precipitated by the addition in the cold of 1 ml of 3.56N $HClO_4$ and 4.5 ml of 95% ethanol to the test sample. The necessity of addition of alcohol is determined by the fact that the RNA preparations were considerably depolymerized and were poorly precipated by acid without alcohol. After 15 minutes the residue was removed by filtration and the filtrate was neutralized with concentrated KOH. The precipitate of $KClO_4$ was removed by filtration, the filtrate was diluted and the absorption in UV light was determined on the SF-4 spectrophotometer. The results are given in Table 1.

TABLE 1. Enzymic Decomposition of RNA in Various Buffer Solutions Values of Extinction at 260 mμ. ΔE–Increase in Extinction During the Time of Incubation. Dilution: 1 ml Filtrate + 9 ml H_2O. Calculated in Terms of 1 mg Bone Marrow in the Test Sample

No. of RNA preparation and no. of experiments	Index	with tris buffer		with ortho-phosphate		with pyro-phosphate	
		I*	II*	I	II	I	II
№ 1	E	0,580	0,670	1,590	1,790	1,340	1,380
Mean of 5 experiments	ΔE	—	0,090	—	0,200	—	0,040
№ 2	E	0,535	0,633	0,550	0,695	0,447	0,512
Mean of 6 experiments	ΔE	—	0,098		0,145	—	0,065
Mean with preparations 1 and 2	ΔE	—	0,094	—	0,162	—	0,052

* I–before incubation; II–after incubation

The figures in Table 1 showing the breakdown of two different RNA preparations by bone marrow enzymes show that decomposition of RNA in orthophosphate is roughly 2-3 times greater than in the other buffer solutions. This does not exclude the possibility of a reaction of orthophosphorolysis of RNA side by side with its hydrolytic decomposition. By the use of the paper chromatography method, however, we were unable to show

the formation of nucleoside diphosphates during the enzymic breakdown or RNA in orthophosphate buffer solution. It is clear from Table 1 that the ratios between the magnitudes of the decomposition of the two different RNA preparations remain quite close to each other in the different buffer solutions, in spite of the fact that the absolute values of the extinctions in the experiments with preparations No. 1 and No. 2 differ considerably from each other. The absolute values of the extinction in the control tests were dependent on the degree of depolymerization of RNA. It is evident that within certain limits the depolymerization of RNA does not lower its liability to enzymic decomposition.

The action of bone marrow extracts on different ribonucleotides. Having admitted the possibility of the enzymic synthesis of polynucleotides from nucleoside diphosphates by the reaction of retrophosphorolysis, we used preparations of ADP and ATP and also free nucleotides which we isolated from the bone marrow of rabbits as initial substances for this reaction. The action of aqueous extracts of bone marrow on various nucleotides was investigated in tris buffer solution, pH 7.0, in the presence of Mg^{++} ions, and with RNA "priming". The amount of bone marrow added to the test samples was such that it did not lead to appreciable disamination of the adenine nucleotieds. No displacement of the absorption maximum took place after incubation. The experimental test samples were incubated for 60 minutes at 37°. After incubation (and in the controls – without incubation) the samples were analyzed by the technique described above for the experiments to investigate ribonuclease activity. The presence or absence of a reaction of polymerization of nucleotides was determined by the change in the magnitude of extinction of the acid-soluble fraction during incubation. In Table 2 are presented the results of experiments showing the action of bone marrow extracts on different nucleotides.

TABLE 2. Action of Bone Marrow Extracts on Various Ribonucleotides E_1: Mean Value of Extinction in Test Samples Without Incubation; ΔE: Difference Between Mean Values of Extinction of Test Samples with and without Incubation. Dilution: 1 ml Filtrate + + 9 ml H_2O; Calculated in Terms of 1 mg Bone Marrow in the Test Sample

Nucleotides	No. of experiments	E_1	ΔE
ADP	2	2,540	0,000
ATP*	8	3,920	—0,120
Free bone marrow nucleotides + ATP	2	4,400	—0,120

* The ATP preparation contained 30% ADP

The figures in Table 2 show that no synthesis of polynucleotide from ADP took place. This is against the hypothesis of the presence of a retrograde reaction of phosphorolysis of RNA in the bone marrow. The slight decrease in the free nucleotides in the experiments with ATP is evidence in favor of the hypothesis that nucleoside triphosphates are the immediate precursors of RNA in the bone marrow.

Changes in the ribonuclease activity of the bone marrow after x-ray irradiation. According to data in the literature [4], in radiation sickness in certain conditions a 3-5-fold increase has been observed in the ribonuclease activity of the bone marrow, but in other conditions this activity was unchanged. An orthophosphate buffer solution was not used in the investigation.

In the experiments described in Table 3, the enzymic breakdown of RNA was investigated in orthophosphate buffer by the technique described above, with 0.1 mg bone marrow per test sample. Twenty-four hours after local irradiation of the limb of the rabbit in a dose of 2000 r the enzymic breakdown of RNA by the bone marrow extracts in orthophosphate buffer fell on the average by 58%.

Change in the Content of Acid-Soluble Purine and Pyrimidine Compounds in the Bone Marrow at Different Periods after Irradiation

There is no information in the literature regarding the determination of the total bone marrow nucleotides at different periods after local irradiation. Our findings are given in Table 4.

TABLE 3. Enzymic Breakdown of RNA in Orthophosphate Buffer Solution by Bone Marrow Extracts in Normal Conditions and 24 Hours after Irradiation in a Dose of 2000 r ΔE_1: Increase in Extinction after Incubation for 60 Minutes in Normal Conditions; ΔE_2: Increase in Extinction after Incubation for 60 Minutes after Irradiation. Values of Extinction – Mean of Parallel Determinations at 260 mμ. Dilution: 1 ml Filtrate + 9 ml H_2O. Calculated in Terms of 1 mg Bone Marrow in the Test Sample.

Relative normal*			Irradiation			ΔE_2 as % of ΔE_1
Before incubation	After incubation	ΔE_1	Before incubation	After incubation	ΔE_2	
3,480	4,480	1,000	3,480	3,920	0,440	44
3,340	3,640	0,300	3,340	3,460	0,120	40

* Relative normal: Corresponding data for the unirradiated limb of an animal exposed to local irradiation.

TABLE 4. Total Quantity of Purine and Pyridine Compounds in Phosphate-Chloric Acid Extracts of Rabbit's Bone Marrow in Normal Conditions and at Different Periods after Local X-Ray Irradiation in a Dose of 2000 r. [The quantity of purine and pyrimidine compounds is expressed as values of extinction at the points of its maximum (255 mμ), calculated in terms of 1 g bone marrow in 100 ml of extract.]

Time after irradiation	No. of experiments	Normal	Irradiation	Time after irradiation	No. of experiments	Normal	Irradiation
10 min	6	0,407	0,417	3 days	1	0,568	0,288
2 hr	3	0,437	0,468	4 days	1	0,542	0,300
7 hr	2	0,379	0,378	5 days	1	0,298	0,185
16 hr	1	0,354	0,275	7 days	1	0,444	0,330
20 hr	3	0,406	0,319	9 days	3	0,323	0,296
24 hr	5	0,442	0,301				

The results in Table 4 show that there is only a small and brief increase in the quantity of nucleotides two hours after irradiation. The maximum decrease in the nucleotide content takes place three days after irradiation. Attention is drawn to the reversible character of these disturbances: nine days after irradiation the nucleotide content is approximately normal again.

Autolytic Breakdown of the Nucleic Acids of the Bone Marrow in Normal Conditions and at Various Periods after X-Ray Irradiation

There are no investigations reported in the literature on the study of the autolysis of the nucleic acids (NA) of the bone marrow at different periods after irradiation. In our experimental conditions we determined principally the breakdown of NA and not of proteins, for the absorption spectra of phosphate-chlorate extracts in UV light in our experiments were characteristic of nucleotides and not of the products of protein breakdown.

It will be seen from Table 5 that within a few hours after irradiation there is a sharp fall in the intensity of autolysis of NA. There is a characteristic reversibility of inhibition of autolytic decomposition of NA throughout the whole period of the investigation – during the nine days after irradiation. The progressive inhibition of autolysis at the latest periods of the investigation indicates that a low level of autolytic decomposition of NA is maintained much longer than nine days.

TABLE 5. Autolytic Breakdown of NA or Rabbit's Bone Marrow in Normal Conditions and after Local Irradiation with X-Rays in a Dose of 2000 r. (Extinction of acid extracts at points of maximum value (255 mμ), calculated in terms of 1 g bone marrow in 100 ml extract. E_1 : growth in extinction after incubation fro 60 minutes in normal conditions; E_2 : growth of extinction after incubation for 60 minutes after irradiation

Time after irradiation	No. of experiments	Normal			Irradiation			ΔE_2 as % of ΔE_1
		Before incubation	After incubation	ΔE_1	Before incubation	After incubation	ΔE_2	
10 min	6	0,391	0,472	0,081	0,401	0,483	0,082	101
2 hr	3	0,437	0,582	0,145	0,469	0,590	0,121	84
7 hr	2	0,379	0,509	0,130	0,378	0,440	0,062	47
16 hr	1	0,354	0,455	0,101	0,275	0,305	0,030	30
20 hr	2	0,394	0,516	0,122	0,296	0,348	0,052	43
24 hr	2	0,428	0,602	0,174	0,312	0,411	0,099	57
3 days	1	0,568	0,676	0,108	0,288	0,326	0,038	35
4 days	1	0,542	0,700	0,158	0,300	0,377	0,077	49
5 days	1	0,298	0,410	0,112	0,185	0,239	0,054	48
7 days	2	0,444	0,517	0,073	0,330	0,354	0,024	32
9 days	2	0,472	0,532	0,060	0,436	0,444	0,008	12

It was found that a tenfold decrease in the dose of irradiation (200 r instead of 2000 r) led to a decrease in the content of free nucleotides (by 10-20%) and also to a decrease in the degree of autolysis of the nucleic acids (by 20-25%) in the bone marrow during the hours immediately after irradiation.

DISCUSSION OF THE RESULTS

The changes in the content of acid soluble purine and pyrimidine compounds in the bone marrow after irradiation are evidently due to the following causes: 1) a transient increase in the content immediately after irradiation is probably due to acceleration of autolysis of NA at this time; 2) the subsequent considerable decrease in the content of nucleotides may be explained by the increased permeability of the cells and vessels of the bone marrow after irradiation; 3) it is possible that the change in the nucleotide content of the tissue after irradiation is to some extent due to a change in the adsorption binding of nucleotides by proteins, for we know from the literature that the adsorption properties of proteins may change significantly after irradiation.

The observed decrease in the content of nucleotides in the bone marrow after irradiation cannot be attributed to a change in the water content, which is not significantly altered under the influence of irradiation [5]. The decrease in the concentration of nucleotides in the bone marrow after irradiation may evidently largely account for some of the principal physiological changes in the bone marrow observed after irradiation, namely: 1) inhibition of nucleic acid synthesis, since nucleotides are the precursors of nucleic acids; 2) inhibition of oxidative phosphorylation, since nucleotides take part in this process; 3) inhibition of mitotic division of cells, since cell division evidently takes place at the expense of the energy of the high-energy phosphate bonds of the nucleotides.

In the figure the changes in the content of free purine and pyrimidine compounds are compared with the changes in the intensity of autolysis of NA of the bone marrow after irradiation. It will be clear from comparison of the curves in this figure that there is a certain analogy between the changes in the nucleotide content and the intensity of autolytic decomposition: in both cases a considerable fall is observed in the first few hours.

From the point of view of radiobiology it is of great interest to investigate the early, profound and irreversible biochemical changes after irradiation. It must therefore be pointed out that radiation injury leads to more considerable changes in the autolytic decomposition of NA than in the content of free nucleotides. Only seven hours after irradiation the fall in the intensity of autolysis of NA is more marked than the maximum fall in the nucleotide content observed three days after irradiation. Within the limits of the period of investigation, the fall in the nucleotide content bears a temporary, transient character, whereas the inhibition of the autolytic decomposition of NA is irreversible.

The inhibition of autolysis evidently results from three causes: 1) a decrease in the content of NA in the bone marrow [6,7,]; 2) a decrease in the activity of the nucleases in the tissue, which we discovered in experiments on the reduction in enzymic decomposition or RNA by bone marrow extracts 24 hours after irradiation;

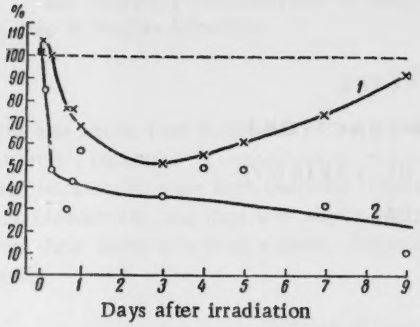

Fig. Changes in the intensity of autolytic decomposition of NA and in the content of acid-soluble purine and pyrimidine compounds in the bone marrow of a rabbit after local x-ray irradiation in a dose of 2000 r. 1) Content of acid-soluble purine and pyrimidine compounds; 2) intensity of autolytic decomposition of NA.

and 3) a change in the composition of NA after irradiation [8,9]. It is possible that after irradiation there is an intensified decomposition of those nucleic acids whose ready decomposition by nucleases is determined by the specific features of their structure; molecules of NA remain, the composition of which determines their smaller liability to autolytic decomposition. By acting as "primers" in the synthesis of new NA, these molecules bring about the further synthesis of NK which is also comparatively resistant to autolytic decomposition.

The decrease in the autolytic decomposition of NA after irradiation is evidently a reflection of their lowered physiological activity in the cell, and may be the cause of the slowing down of those profound structural changes in the cell which are necessary for mitotic division. The irreversible fall in the autolytic decomposition of NA after irradiation is a possible cause of the development of leukemia and cancer at remote periods after irradiation.

Some authors [10-12] did not observe a phase of inhibition of autolysis of proteins after irradiation, for the pattern of change of autolysis evidently depends on the type of tissue, the substrate decomposed during autolysis (RNA, protein) and on other conditions.

SUMMARY

Changes in the content of acid-soluble purine and pyrimidine compounds and in the intensity of autolysis of nucleic acids in the bone marrow are sensitive reactions to irradiation. These changes may be detected after local irradiation in a dose of 200 r or less.

It has been shown that the combination of acid extraction with preliminary homogenization of the tissue in orthophosphate buffer solution in the cold facilitates a more complete extraction of the purine and pyrimidine compounds of the tissue than ordinary acid extraction.

We express our gratitude to N. A. Kirpichnikova for her help with this work.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

AN ELECTROPHORETIC STUDY OF THE PROTEIN FRACTIONS OF Bacillus Breslau AND Escherichia coli AND OF VARIANTS OF Escherichia coli OBTAINED BY ASSIMILATION OF PRODUCTS OF Bacillus Breslau

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The attention of micorbiologists has recently been attracted to the investigation of variation of microorganisms by means of the cultivation of one species of microorganism on the metabolic products or on the disintegration products of another species. When grown in this way, a strain of one species acquired the properties of that species on whose products it was cultivated.

By cultivation of Escherichia coli on media containing autolyzates of Bacillus Breslau, variants of E. coli were obtained which, in their serological properties, virulence, antigenicity and sugar fermentation, very closely resembled the "inducing" culture, i. e. B. Breslau [1,2].

In previous work [3,4] we found that the above-mentioned cultures differ from the leading culture in their content of nucleic acids and their nuclease activity. In this connection we considered that it would be of interest to isolate the proteins from the original strains and variants and to study some of their properties, since it is difficult to imagine changes caused by controlled variation without changes in the specific properties of the proteins in these microorganisms. The present research is devoted to the electrophoretic investigation of some of the protein fractions isolated from the original strains E. coli 163 and B. Breslau 353, and from variants E. coli 125 and 60. There are very few such investigations reported in the literature, and such work as there is mainly concerns the study of the electrophoretic properties of the proteins of tubercle bacilli.

For instance, Seibert and Watson [5] isolated the protein fractions from a human strain of Mycobacterium tuberculosis and investigated their electrophoretic mobility. Seibert, Pedersen and Tiselius [6] studied the electrophoretic properties of three protein fractions isolated from tubercle bacilli and found that one fraction consisted of three components, and the other two fractions of two components. Chargaff and Moore [7] isolated a nucleoprotein from Bacillus tuberculosis avium, and showed by electrophoretic investigations that it consists of a single component.

EXPERIMENTAL SECTION

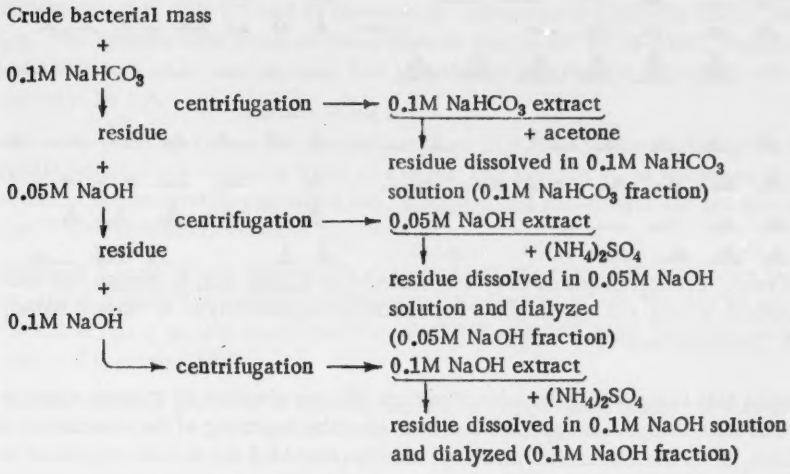
Characteristics of the strains investigated. E. coli 163, S form, serologically typical, fermented glucose, lactose, maltose and mannitol with the formation of acid and gas, did not ferment saccharose, produced indole and did not form H_2S. B. Breslau 353, S. form, serologically typical, fermented glucose, maltose and mannitol with formation of acid and gas, did not ferment saccharose lactose, did not produce indole, formed H_2S. Variant E. coli 125 gave a diffuse growth with a sediment in broth, was agglutinated only to a titer by B. Breslau antiserum, fermented carbohydrates like B. Breslau but without gas formation, produced neither H_2S nor indole; it was less pathogenic than B. Breslau towards white mice. Variant E. coli 60 in broth gave a diffuse growth

with a residue, was agglutinated to a titer by B. Breslau antiserum and was not agglutinated by other sera. Carbohydrates were fermented as by B. Breslau, but without formation of gas. Neither indole nor H_2S was formed. It was absolutely nonpathogenic towards white mice. Both variants were antigenic and protected white mice againse B. Breslau infection.

EXPERIMENTAL METHOD

The test strains were cultivated on meat-peptone agar at 37° for 18 hours, and then washed off with physiological saline, separated by centrifugation and washed three times with the same solution. The protein fractions were isolated from the fresh bacterial mass thus obtained by means of successive extraction with solutions of sodium bicarbonate, and then with alkali, with mechanical agitation and cooling with ice. Extraction was performed three times with each solvent. Isolation of the fractions was achieved in accordance with the following scheme:

Scheme of Fractionation of Bacterial Mass



The fractions thus isolated were lyophilized and certain of their chemical and physico-chemical properties were studied, but in this communication we shall not discuss these but confine our attention to their electrophoretic investigation. Electrophoresis was carried out by the moving boundary method in 0.1M veronal buffer with pH 8.6, μ 0.1, and in 1/15M phosphate buffer with pH 7.7, μ 0.099, with a current strength of 5-6 ma and a voltage of 120-130 v.

EXPERIMENTAL RESULTS

The results of the investigations are shown in the figure, which illustrates the course of electrophoresis of the various protein fractions in time. On the assumption that in the same experimental conditions, i. e. in veronal buffer (pH 8.6), the protein fractions which we investigated could not be completely divided into their components, we decided to undertake experiments in other condtions too, namely in phosphate buffer at pH 7.7.

It can be seen from the figure that the 0.1M $NaHCO_3$ protein fraction from the original strains and the variant E. coli 125 moved as a single peak, but that from the variant E. coli 60 moved as two peaks. We were able to observe obvious separation of the components in the last case one hour after the beginning of the experiment. Subsequently the slow component lagged further and further behind. This protein fraction was a nucleoportein in its chemical properties and in its N/P ratio, which was 4.0-5.0. These observations show that the nucleoprotein fraction from both the original strains and variant 125 cannot be distinguished by its electrophoreric properties in veronal or phosphate buffers, but that form variant 60 can be distinguished from the original strains and variant 125.

Let us now consider the electrophoretic properties of the 0.05M NaOH protein fraction. In veronal buffer the 0.05M NaOH protein fraction from both the original strains and the variants moved as two peaks. In spite of

the fact that the bulk of the protein consists of two components in E. coli 163 and B. Breslau 353, the degree of difference was considerably diminished. Both components were approximately alike in their electrophoretic mobility.

From the analysis of the data obtained during investigation of the electrophoregrams of the 0.05M NaOH protein fraction in phosphate buffer, it can be seen that in the original strains and in variant 60 this protein fraction moved as it did in veronal buffer, in the form of two peaks, and that form variant 125 moved as three peaks.

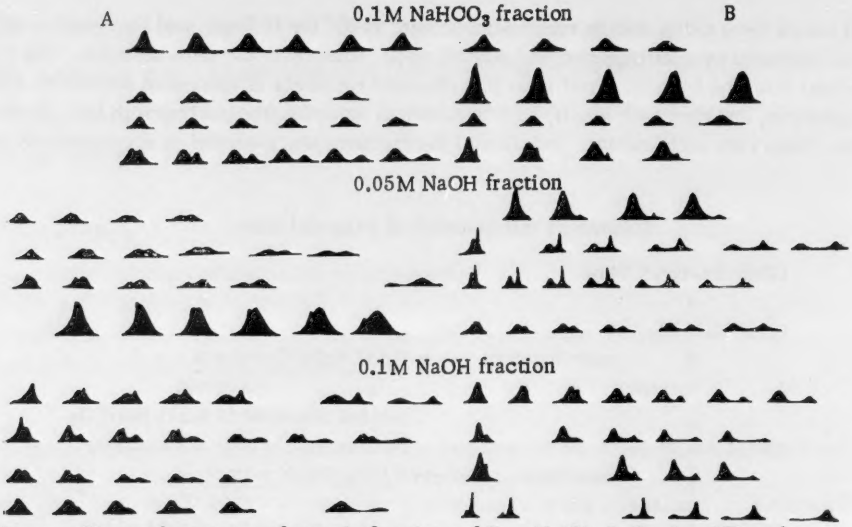


Figure. Electrophoregrams of protein fractions of E. coli 163, B. Breslau 353 and variants E. coli 125 and 60 (from top to bottom respectively). A: veronal buffer; B: phosphate buffer.

A clear separation into two peaks in the case of variant 125 was observed 15 minutes after the beginning of the experiment. The third component appeared one hour after the beginning of the experiment and moved as a small peak which, in its electrophoretic mobility, closely resembled the second component of this fraction.

From a comparison of the electrophoretic data obtained for the 0.05M NaOH protein fraction when investigated in veronal and phosphate buffers, it can be concluded that the 0.05M NaOH protein fraction consists of two components in the original strains and in variant 60. The electrophoretic dissociation of the 0.05M NaOH protein fraction of variant 125, when investigated in phosphate buffer, differs from the electrophoretic dissociation taking place in veronal buffer in the number of components. The reason for this is that in veronal buffer (pH 8.6) the values of the electrophoretic mobility of the individual proteins of this fraction are very close or even identical. By modifying the experimental conditions, i.e. by conducting the electrophoresis of the test proteins in phosphate buffer (pH 7.7), we found three components.

It must be pointed out that whereas the two components were situated close to each other during electrophoresis of the proteins of the original strains in veronal buffer, when the investigation was carried out in phosphate buffer they were clearly demarcated from each other even at the beginning of the experiment. This difference may probably be due to the fact that in the experiments with veronal buffer (pH 8.6) the particles of the individual proteins interact with each other, and therefore migrate together, which is not observed in phosphate buffer (pH 7.7). When the electrophoresis was carried out in veronal buffer, the 0.1M NaOH protein fraction from the original strain E. coli 163 and the variants E. coli 125 and 60 moved as two peaks, and that from the original strain B. Breslau 353 as three peaks. Examination of the electrophoregrams of this fraction obtained with the use of phosphate buffer shows that both the original strains and the variants E. coli 125 and 60 consist of two components.

We thus see that, in accordance with the electrophoretic mobility of their 0.1M NaOH protein fraction, the variants E. coli 125 and 60 resemble the original strain E. coli 163 and differ from the "inducing" strain B. Breslau 353.

DISCUSSION OF THE RESULTS

It is clear from the results described that during the controlled variation of E. coli profound changes take place in the properties of its proteins. At the same time our results demonstrate the absence of full identity of variants with the "inducing" strain B. Breslau, thus refuting the idea of the conversion of the microorganism undergoing variation into an inducing species.

The conclusion that during the "direction of properties" profound changes take place in the proteins of the micororganism was also reached by Rashba and Kaganskaya [8], who studied the chemical composition and the properties of the nucleoproteins of these same strains. Belozerskii and co-workers [9] studied the chemical composition and immunological properties of protein fractions isolated from original and deliberately varied strains, and they also concluded that a radical, qualitative transformation of the proteins of the microorganism undergoing variation takes place. The further investigation of the individual components of the protein fractions thus obtained will permit a more detailed study of their properties.

SUMMARY

A study was carried out of the electrophoretic properties of protein fractions isolated from E. coli 163, B. Breslau 353 and from variants of E. coli 125 and 60 obtained by cultivation of E. coli in media containing autolyzates of B. Breslau. The cultures were grown on meat-peptone agar at 37° for 18 hours. Protein fractions were isolated from bacterial masses by successive extraction with sodium bicarbonate and alkali with mechanical agitation and refrigeration on ice.

Electrophoresis was carried out by the mobile boundary method in 0.01 M veronal buffer (pH 8.6), μ 0.1 and in a 1/15 M phosphate buffer at pH 7.7 and μ 0.099 at 5-6 ma and 120-130 v. It was shown that the 0.1 M $NaHCO_3$ protein fraction of the original E. coli strain 163, B. Breslau 353 and variant 125 are similar in their electrophoretic behavior, which is however different in variant 60.

In the original strains of E. coli 163, B. Breslau 353 and variant 60 the 0.05 M NaOH protein fraction consists of two components, and in variant 125 - of three components. Variants of E. coli 125 and 60 retain the same components of the 0.1 M NaOH protein fraction as the original E. coli 163 strain but differ in this respect from the "inducing" strain of B. Breslau 353.

In conclusion I express my deep gratitude to Prof. E. Ya. Rashba for guidance in the conduct of this research.

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THE ROLE OF ADENOSINETRIPHOSPHATE IN THE RHYTHMIC MOVEMENT OF THE CILIA IN INFUSORIA

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The movement of infusoria is effected by the harmonized rhythmic movement of numerous cilia or of their derivatives. In spite of the presence of a regulatory apparatus which ensures the integration and coodination of the complex motor system of the infusoria [1-4], there is reason to believe that each individual cilium has its own mechanism responsible for its movement [1, 5].

The investigations of Aleksandrov and Arronet [6], carried out on cell models of the ciliated epithelium of the frog, show that the source of energy for the work of cilia in the tissues of multicellular animals is ATP. The importance of ATP for the movement of the flagellae of unicellular organisms was shown earlier still by Hoffmann-Berling [7-9]. The researches at first glance seemed to indicate that the biochemical processes lying at the basis of the activity of the cilia in the infusoria are the same as those established for the epithelial cilia of the Metazoa. As a result of further investigations, however, it has been shown that the presence of ATP is not essential for the contraction of the stem of Vorticella [10]. Consequently, the biochemistry of the contractile structures of the Protozoa, which appeared in the process of evolution independently from the contractile structures of multicellular animals, may present its own specific features. This served as the basis for the experimental verification of the role of ATP in the movement of cilia and other organella of movement of infusoria, phylogenetically developing from cilia (membranelles and cirri).

EXPERIMENTAL METHOD

In order to elucidate the role of ATP in the ciliary movement of infusoria, we made cell models from Protozoa, using for this purpose Hoffmann-Berling's saponin method [10], which was slightly modified. The basic experiments were carried out on Spirostomum ambiguum Ehrb. and Euplotes patella Ehrb. In the models prepared from Spirostomum we studied the activity of the cilia, and in Euplotes the work of the membranelles and cirri. Clones of these infusoria were cultivated on Losina-Losinsky's mineral medium [11], as adopted for Paramecium caudatum [12].

Before the experiment began the Protozoa were washed in bidistilled water. Next, they were placed in a cytolytic solution (CS), cooled to 0°, of the following composition: 0.12M KCl, 0.01M phosphate buffer, 0.004M Na_2EDTA and 0.5% saponin. The pH of the CS prepared for Spirostomum was 6.5-6.6, and for Euplotes 6.9-7.0 (digitonin may be used instead of saponin). In the experiments with Euplotes, positive results are obtained only if the concentration of saponin (or digitonin) in the CS does not exceed 0.15%. The cytolytic solution with the protozoa is kept in the refrigerator at T=0°.

Substances of the saponin group are known to possess well-marked cytolytic properties: they quickly inactivate enzymes and destroy the cell. EDTA, which does not diminish the strength of action of saponin, delays the disintegration of the protozoa and prevents the onset of an irreversible contraction of the contractile protoplasmic structures [10].

Infusoria which had been kept for 30-45 minutes in CS cooled to 0° were then transferred to an experimental solution (ES) containing 0.12M KCl and 0.01M phosphate buffer (at pH 6.5 for Spirostomum and 7.0 for Euplotes). The influence of the various substances on the cilia, membranelles and cirri of the cytolyzed protozoa was investigated in ES at room temperature (16-18°). All the solutions (including CS and ES) were prepared in bidistilled water, for even traces of the heavy metals may prevent the contraction of the contractile proteins of the model [13,14]. The ATP used in the experiments was prepared by the Ivanovo Meat Combine. Solutions of ATP were prepared in ES and neutralized with 1N NaOH.

EXPERIMENTAL RESULTS

In protozoa kept for 30-45 minutes in CS, the organella of movement—the cilia, membranelles and cirri—were mobile. The clearly outlined macronucleus of Spirostomum ambiguum was readily visible throughout its whole length. The endoplasm was coagulated and therefore brightly luminescent in the dark field of the microscope; the ectoplasm appeared more transparent. Some of the infusoria were partially or completely destroyed. Similar changes, suggesting death of the protozoa, were also clearly distinguished in the cytolyzed Euplotes patella.

If the infusoria were transferred from CS into ES or into Losina-Losinsky's medium, then neither in Spirostomum nor in Euplotes did restoration of the vital functions take place. The cilia remained immobile. Moveover, 10-15 minutes later, disintegration of the protozoa began to occur, and its course was especially rapid in Spirostomum. The addition of EDTA had a very slight restraining action on the commencing disintegration of the unicellular organisms.

ATP in a range of concentrations from $0.5 \cdot 10^{-3}$ to $1 \cdot 10^{-2}$M did not bring about restoration of the rhythmic movement of the cilia of the cytolyzed infusoria. If, however, even only a small amount of Mg^{++} ($1 \cdot 10^{-3}$ g·ion/liter Mg^{++}) was added along with the ATP to the ES, the organella of movement of the models showed revival of their activity, outwardly resembling the working of the cilia of living infusoria. Under these circumstances the models of Spirostomum remained absolutely immobile, for coordination between the cilia was completely absent. After the addition of Mg^{++} ions and ATP to the ES containing cytolyzed Euplotes, not only did recovery of the working of the organella of movement take place, but the organisms also began to crawl and swim.

This happened because the peristomal membranelles, which are mainly responsible for the movement of the living Euplotes, contracted relatively harmoniously in the models. In this case we may speak of residual regulation, preserved in the motor system of the cytolyzed protozoa. It is evident that coordination between the peristomal membranelles is effected not only by metachronal impulses, but also as a result of a purely mechanical connection between these organella. A more precise answer to the question of the mechanism of the residual regulation may, however, be obtained only after additional research.

The cirri of the models of Euplotes contracted very energetically but disorderly in the presence of Mg^{++} and ATP. The activity of the organella of movement of the cytolyzed Spirostomum ambiguum continued for 40-60 minutes, and that of Euplotes patella for 60-90 minutes. The models of Spirostomum and Euplotes were poorly preserved in CS even at 0°. After they had been kept for 1.5 hours in CS, it was very difficult to induce activity in the cilia, membranelles and cirri of the cytolyzed infusoria. At this period the Spirostoma were completely destroyed.

In ES at room temperature ciliary movement may be induced in the models for 10-15 minutes. After this period the addition of Mg^{++} and ATP in any concentrations does not renew the activity of the organella of movement. After the same period of time the Spirostoma were partially or totally destroyed. Only if the cytolyzed protozoa were transferred soon enough from the CS into the ES with Mg^{++} and ATP could prolonged activity of the organella of movement be produced.

The influence of various factors on the activity of the cilia, membranelles and cirri of the models of infusoria was investigated. The most favorable value of the pH of the experimental solution for activity of the cilia of the cytolyzed Spirostoma was 6.5-6.6. Only very slight changes in the pH of the medium in either direction caused a sharp decrease in the duration of activity of the organella of movement or totally suppressed the ciliary movement. The models of Euplotes were less sensitive to this factor; activity of their membranelles and cirri was observed even at pH 6 and 8 of the ES. In the range of pH 6.5-7.3, the Euplotes models were

motile. In a neutral medium the organella of movement of the cytolyzed Euplotes retained their activity for 1.5 hours.

If the K^+ content of the experimental solution was increased to 0.25-0.30, then ATP ceased to cause motility of the cilia, membranelles and cirri in the models. A decrease in the K^+ concentration in the ES to 0.01M hardly affected the activity of the organella of movement of the cytolyzed infusoria. Transient motility of the cilia in the Spirostoma models appeared even when the only K^+ in the composition of the experimental solution could be completely replaced by Na^+ (0.12M). But if Li^+ was added instead of K^+, it was not possible to induce ciliary activity in the cytolyzed protozoa.

The activity of the organella of movement of the models was of maximum duration when the ES contained $2 \cdot 10^{-3}$-$2 \cdot 10^{-2}$ g·ion/liter Mg^{++}. Higher or lower concentrations of this cation led to a decrease in the period of ciliary activity. The minimum quantity of Mg^{++} to permit working of the model was $0.5 \cdot 10^{-4}$ g·ion/liter; Ca^{++} could not be substituted for Mg^{++}.

Ciliary activity in the cytolyzed Spirostoma was caused by ATP in a concentration range of $5 \cdot 10^{-4}$ to $1 \cdot 10^{-2}$M. The minimum concentration for Euplotes was $1 \cdot 10^{-3}$M. If, instead of ATP, the cytolyzed protozoa were treated with $1 \cdot 10^{-5}$-$1 \cdot 10^{-2}$M ADP, AMP or inorganic pyrophosphate, no motility of the cilia, membranelles and cirri was observed. ATP itself, and no other phosphorus-containing substance of similar structure, was essential in this case.

Pyrophosphate in concentrations of over $1 \cdot 10^{-3}$ mole/liter suppressed the ciliary motility of the models. ADP and AMP did not lower ciliary activity even in concentrations of $1 \cdot 10^{-2}$M. Na_2EDTA (0.005M), by combining with Mg^{++}, caused the rapid immobilization of the organella of movement of the cytolyzed infusoria. Models washed free from trilon B, in the presence of Mg^{++} and ATP renewed their ciliary activity.

It must be pointed out that living Spirostoma and Euplotes, in solutions of 0.005M Na_2EDTA prepared in bidistilled water, retained their motility for about two hours. In general, the motor system of the models of the infusoria was more sensitive to various chemical agents ($NiSO_4$, formalin, etc.) than the motor system of the living infusoria. Heparin and germanin, in concentrations of $1 \cdot 10^{-5}$, greatly weakened, and in higher concentrations ($1 \cdot 10^{-4}$-$1 \cdot 10^{-2}$M) completely suppressed the activity of the cilia of the models. Trypan blue immobilized the membranelles and cirri of cytolyzed Euplotes in a concentration of $1 \cdot 10^{-4}$M, and the cilia of the cytolyzed Spriostoma in a concentration of $5 \cdot 10^{-5}$M. Strophanthin and diethylstilbestrol ($1 \cdot 10^{-6}$-$1 \cdot 10^{-4}$M) had no appreciably influence on the activity of the organella of movement.

In the process of preparation of the models a number of Spirostoma were destroyed, as a result of which fragments of the pellicule of different sizes, with spheres and granules of endoplasm, remained in the CS. Under the influence of $1 \cdot 10^{-3}$M ATP and $1 \cdot 10^{-3}$g·ion/liter Mg^{++} in the ES, the cilia remaining on the fragments of half-disintegrated infusoria renewed their activity, which lasted for 15-40 minutes. Cilia completely detached from the model were capable of contraction under the influence of ATP. The following experiment was carried out to prove this.

CS, containing Spirostoma models ready prepared for the work, was transferred from the refrigerator to room temperature (T=18°). After 5-7 minutes ATP was added to the CS in sufficient quantity to bring its concentration in the ES up to $1 \cdot 10^{-2}$M; by means of a fine glass pipette the infusoria which were beginning to disintegrate under these conditions were collected and injected forcefully into ES containing $1 \cdot 10^{-3}$g·ion/liter Mg^{++} and $1 \cdot 10^{-3}$M ATP. Under a microscope fitted with a dark field system it could be seen that as a result of this procedure the Spirostoma were totally destroyed; in the field of vision there remained large numbers of isolated cilia, which performed rhythmic, wavelike contractions. Their activity lasted 10-15 minutes. This experiment showed that ciliary motility develops by the direct action of ATP on their contractile proteins.

In concentrations of 0.01-0.001% $NiSO_4$ immobilized Spirostomum, Euplotes and Paramecium. The ciliary motility of the protozoa was maintained under these conditions, however, for 1-3 hours. $NiSO_4$ apparently suppressed the formation of the spontaneous metachronal impulses which coordinate the activity of the motor system of the infusoria as a whole, but did not disturb the mechanisms responsible for the motility of the cilia. If living paramecia were placed in a 0.02% solution of phosphacol, a very curious process was observed: cilia began to be detached from the infusoria after 7-10 minutes, and each isolated cilium continued to contract rhythmically for 1-3 minutes. This experiment, and those described above, in our opinion prove convincingly

that each individual organellum of movement possesses an independent mechanism of movement, as has been postulated by Worley [1].

DISCUSSION OF THE RESULTS

Our findings show that with the aid of Hoffmann-Berling's saponin method it is possible to obtain cell models of infusoria for investigation of the role of ATP in ciliary activity.*

It has been found [15] that to obtain contraction of a model of a muscle fiber the experimental solution must contain K^+ (optimally 0.12M) and Mg^{++} in addition to ATP. The same conditions are essential for the activity of the cilia, membranelles and cirri of cytolyzed protozoa. Heparin, germanin, trypan blue and other polythioacids prevent contraction of models of muscle fiber and models of the flagella of trypanosomes [8,14,16]. These substances in the same concentrations cause arrest of motility of the cilia, membranelles and cirri of cytolyzed infusoria. In the presence of high concentrations of pyrophosphate, ATP does not cause contractions of models of muscle fibrils [14]; the organella of movement in this case also are not rendered active. Hence it may be concluded that the motility of the cilia, membranelles and cirri of the Ciliata is based upon biochemical processes similar to those which cause contraction of the muscle fiber. However, whereas the model of a muscle cell contracting under the influence of ATP does not possess the power of relaxation and therefore requires special external action (for example, treatment with salyrgan) in order to abolish the state of contraction of the muscle fibril, the model of the cilia is capable both of contraction under the influence of ATP and of relaxation. The cilia therefore pass into a state of motility and this lasts until irreversible changes take place in their structure. Consequently, the contractile proteins of the cilia, membranelles and cirri possess certain properties which are specific for these structures. In no case were we able to induce contractions of the cilia of the models without their subsequent relaxation.

In muscle cells the energy liberated by the decomposition of adenosinetriphosphate is utilized for the act of contraction [16], but in the stem of Vorticella it is utilized for the act of relaxation [10]. It is evident that ATP is the source of energy for both working phases of the motor organella of the cells—cilia and flagella, i. e. for both their contraction and their relaxation. This accounts for the prolonged motility of the models of flagella and cilia in the ES during the action of ATP (in presence of Mg^{++}).

There is now no doubt that each individual cilium of the infusoria possesses an independent mechanism, provided with its ATP, and that it is therefore capable of performing the whole act of rhythmic movement independently of other cilia. The movement of infusoria may take place only in consequence of a special coordinating apparatus (or apparatuses) which integrate and regulate the work of the many cilia in the interests of the unicellular organism as a whole.

SUMMARY

Models of ciliates (Spirostomum ambiguum Ehrb. and Euplotes patella Ehrb.) were prepared by the Hoffmann-Berling saponin method for studying the biochemical mechanism underlying ciliary movement.

Experimental data show that ATP is an energy donor for ciliary movement. ATP cannot be substituted by ADP, AMP or an inorganic pyrophosphate. Similar experimental conditions are necessary for the activity of the model cilia and for the contraction of the myofibril model. Each cilium of any infusorian has its own mechanism which produces ciliary motility.

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* V. Ya. Aleksandrov and N. I. Arronet (Botanical Institute AN SSSR, Leningrad) independently of us obtained cell models of the infusorian Paramecium caudatum by means of Hoffmann-Berling's saponin method and demonstrated the role of ATP in the ciliary motility of this protozoan (personal communication from V. Ya. Aleksandrov).

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CYTOCHEMISTRY OF THE BIOSYNTHESIS OF RIBONUCLEIC ACID IN EHRLICH'S ASCITES CARCINOMA

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Much research has been done on the localization of the biosynthesis of RNA, but no unanimous conclusion has yet been reached as a result.

Early investigations [1,2] showed that the rate of incorporation of precursors into the nuclear RNA is higher than that into the cytoplasmic RNA, from which it was postulated that RNA is synthesized in the nucleus and from there it passes to the cytoplasm. Barnum and Huseby [3,4], however, from their investigations of the dynamics of incorporation of P^{32} into the RNA of various cell structures, concluded that the precursor of the RNA of the microsomes (the greater part of the RNA of the cytoplasm is connected with the microsomes) is the RNA of the hyaloplasm and not of the nucleus. In their discussions, these workers started from the concepts of the homogeneity of the RNA fractions and of the reversibility of all the reactions of the biosynthesis and transfer of RNA, which can hardly be justified. A recently published series of autoradiographic investigations on cells of different origin [5-10] has yielded evidence in favor of the nuclear origin of the cell RNA. On the other hand Harris [11], who also used autoradiography, came to the conclusion that a considerable part of the nuclear RNA does not pass into the cytoplasm but is destroyed. Despite the considerable value of the above-mentioned investigations, they have one inherent defect: the absence of separation of RNA into its different fractions, which has become technically possible only recently. Above all, this concerns the nuclear RNA, the heterogeneity of which has recently been proved by several researchers. Allfrey and Mirsky [12] separated the nuclear RNA into the fractions RNA_1 and RNA_2, which according to Zbarskii and Georgiev [13-16] correspond to the RNA of the nuclear sap and the RNA of the nucleoli and residual chromosomes. Our investigations showed that by means of phenol treatment it was possible to obtain a number of RNA fractions, evidently entering into the compotition of different types of nucleoprotein complexes [17-19]. The cytoplasm and the nuclear sap contain high-polymer RNA, * low-polymer RNA and RNA which is not extracted by phenol treatment [20,21].

The chromosome-nucleolar apparatus of the nucleus contains RNA extractable with phenol, pH 8.3, 0.14M NaCl and nonextractable RNA (RNA-NE). RNA-HP enters into the composition of the ribonucleoprotein granules

* Abbreviations used in this paper: N-RNA-NE – nuclear RNA-N (RNA of the chromosome-nuclear apparatus not extractable with phenol, pH 8.3, 0.14M NaCl); N-RNA-E – nuclear RNA-E (RNA of the chromosome-nuclear apparatus extractable with phenol, pH 8.3, 0.14M NaCl, but not extractable with phenol, pH 6.0, 0.14M NaCl); NS-RNA-HP – high-polymer RNA of the nuclear sap; C-RNA-HP – high-polymer cytoplasmic RNA; C-RNA-LP – low-polymer cytoplasmic RNA; C-RNA-NE – cytoplasmic RNA not extractable with phenol treatment; RNA-HP – total extractable high-polymer RNA; RNA-LP – total low-polymer RNA; RA – relative activity; RNP – ribonucleoprotein P_{inorg} – inorganic phosphorus.

(ribosomes) of the mitochondria, microsomes, hyaloplasm and nuclear sap [20-25], which are evidently the centers of protein synthesis in the cell, determining the specificity of the protein formed. It is probable that C-RNA-NE is also associated with these granules [20]. Low-polymer RNA is localized to the supernatant fraction. Its role is probably in the transfer of activated amino acids during the biosynthesis of protein. The RNA of the chromosome-nucleolar apparatus (N-RNA-NE and N-RNA-E) also largely enters into the composition of the ribonucleoprotein granules, associated with the nubleonema of the nucleolus and chromatin [26]. According to our new data, it is high-polymer RNA [39].

Taking into consideration the information recently obtained on the cytochemistry of RNA, we decided to investigate the incorporation of P^{32} into the various fractions of the RNA of the cell, using the phenol method of fractionation of the RNA entering into different types of ribonucleoprotein complexes. The results obtained demonstrate that the site of biosynthesis of the high-polymer RNA of the ribosomes is the chromosome-nuclear apparatus.

EXPERIMENTAL METHOD

Experiments were carried out on cells of an Ehrlich's ascites carcinoma, taken on the sixth or seventh day after transplantation.

Conditions of incubation. Ascitic fluid obtained from all the mice taking part in the experiment was mixed with 1/10 its volume of 5% sodium citrate to prevent clotting, $Na_2HP^{32}O_4$ was added in a dose of 10-15 μC/ml ascitic fluid, and the mixture was incubated at 37° aerobically, with agitation, in conical flasks. After different periods of time samples of the material were withdrawn for investigation.

In the principal experiments, after incubation with labeled phosphate in vitro, incubation in vivo was carried out with unlabeled phosphate, in order to determine the possibility of transfer of RNA from some fractions to others. For this purpose the obtaining of the ascitic fluid, the incubation in vitro with P^{32} and the subsequent treatment were done in sterile conditions. After incubation for one hour with P^{32} in the conditions described above, the cells were collected by centrifugation, washed twice with $^1/_{15}$ M phosphate buffer, pH 6.5 to remove radioactive P_{inorg}, suspended in unlabeled ascitic fluid freed from cells to which was added $^1/_{10}$ of its volume of 5% citrate and $^1/_{10}$ its volume of phosphate buffer, and injected into healthy mice—1.2 ml per mouse. The animals were sacrificed after dirrerent intervals of time (from 1 to 20 hours) and the ascites cells were extracted by washing out the peritoneal cavity twice or three times with phosphate buffer.

FRACTIONATION OF THE NUCLEIC ACIDS

Fractionation of the cells obtained was carried out by one of three methods.

Method 1. In one series of experiments we determined the radioactivity only of the RNA and the free nucleotides. For this purpose the cancer cells were washed twice with $^1/_{15}$ M phosphate buffer, pH 6.5, and then extracted five times with cold 5% TCA. The first extract was used for isolation of the nucleotides. The residue was then washed with 70% alcohol, twice with 96% alcohol, once with a 3:1 boiling mixture of alcohol and ether, and twice with ether. The nucleic acids were extracted from the residue with 10% NaCl at 100° for one hour, precipitated with two volumes of alcohol and dried with alcohol and ether.

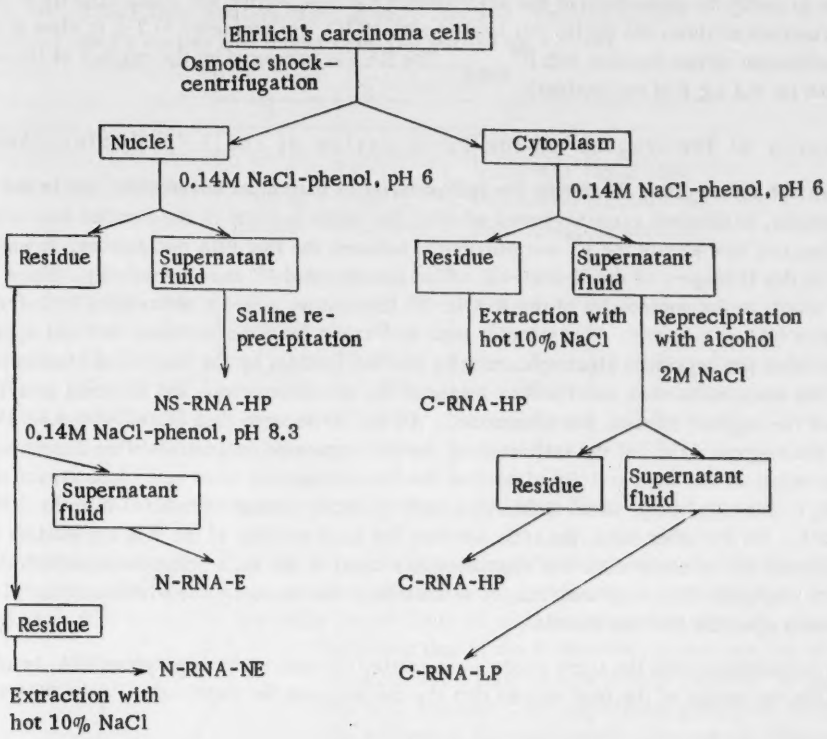
Method 2. In another series of experiments phenol fractionation of the RNA of the ascites cells was carried out [18]. Cells, washed with phosphate buffer, were suspended in 0.14M NaCl. The suspension was treated with an equal volume of phenol at pH 6. After shaking and centrifugation the aqueous phase was collected and the RNA-HP and RNA-LP isolated from it. RNA-HP was precipitated with 2M NaCl, reprecipitated once with 2M NaCl, washed twice with 2M NaCl and then five times with cold 5% TCA, and twice with alcohol and ether. RNA-LP was washed seven times with cold 5% TCA, alcohol and ether.

The intermediate layer was washed once again in a system: phenol, pH 6—0.14M NaCl, and then the phenolic nuclei were isolated from it by a method described previously [18]. The RNA of the phenolic nuclei was fractionated in a system: phenol, pH 8.3—0.14M NaCl. N-RNA-E together with DNA were precipitated with alcohol, and N-RNA-NE was extracted from the residue, previously washed with alcohol, boiling alcohol—ether, with 10% NaCl at 100° for one hour, and precipitated with two volumes of alcohol in the cold. The preparations N-RNA-E and N-RNA-NE were then washed three times with cold 5% TCA and dried with alcohol and ether.

Thus as a result of these procedures, RNA-HP, RNA-LP, N-RNA-E and N-RNA-NE were obtained for analysis.

Method 3. In a third series of experiments further differentiation of the RNA fractions of the cell was carried out (see diagram).

Scheme of Fractionation of RNA of Cells of Ehrlich's Ascites Carcinoma



Isolation of the nuclei was carried out by Smellie's method as modified by Samarina [27]. Ascites cells, washed with $^1/_{15}$M phosphate buffer, pH 8.0, were destroyed by the addition of 12 volumes of iced bidistilled water. The nuclei were precipitated at 600 g for five minutes and washed once with 0.25M saccharose containing 0.1% potassium glycerophosphate. The cytoplasmic fraction was brought up to 0.14M in relation to NaCl, and the nuclei remaining in it were precipitated at 1500 g for 10 minutes, after which it was subjected to phenol treatment, so that fractions C-RNA-HP, C-RNA-LP and C-RNA-NE were obtained [20].

The nuclei were treated with phenol, pH 6.0, 0.14M NaCl, as a result of which NS-RNA-HP was liberated The RNA-E and RNA-NE of the phenol nuclei were isolated from the residue by the usual method [18].

Purification of RNA Nucleotides and Determination of Their Relative Activity

In order to purify the RNA preparations obtained from highly active contaminants, and above all from P^{32}_{inorg}, the method of Mandel and co workers [28] was used. The RNA was hydrolyzed with 0.5N KOH for 18 hours at 37°, after hydrolysis, one sixth of the volume of 70% $HClO_4$ was added during cooling, the resulting precipitate of $KClO_4$ and DNA was removed by centrifugation, and to the supernatant fluid (usually about 5 ml in volume) was added 50 mg of "Norite" activated charcoal, washed with EDTA [29]. The mixture was agitated in the cold for 2-3 hours, after which the Norite was removed by centrifugation and washed 5-6 times with cold 0.5N $HClO_4$ and then twice with cold water.

The washed Norite contained adsorbed nucleotides, which were then eluted with 3-4 ml of a 5:1:4 alcohol 10% NH_3–water mixture at 37° overnight. The eluate was thoroughly freed from Norite particles by repeated centrifugation, filtered, dried, redissolved and placed on a target. After measurement of the radioactivity with an end-type counter, the nucleotides were transferred with 0.5N $HClO_4$ to graduated tubes, and their amount determined spectorphotometrically (by the difference in absorption at 260 and 290 mμ), and in some cases as phosphorus [30].

In order to purify the nucleotides of the acid-soluble fraction, Norite was added directly to the first TCA extract. The number of times the Norite was washed with $HClO_4$ was increased to 7-8 in view of the high degree of contamination of this fraction with P^{32}_{inorg}. The RA was expressed by the number of impulses per minute per μg RNA (or 0.1 μg P of nucleotides).

Determination of the Degree of the Purification of the RNA Nucleotides

Since we did not routinely determine the radioactivity of individual nucleotides, but in the whole fraction of RNA nucleotides, in selected cases we tested whether the whole activity of the fraction was connected with the RNA nucleotides and how evenly the P^{32} was distributed between the four RNA nucleotides. It was particularly important to do this in respect of the N-RNA-NE, which incorporated P^{32} most intensively. Since the amount of this was very small, to the nucleotides of the N-RNA-NE fraction we added a neutralized hydrolyzate of unlabeled high-polymer RNA as a carrier. Two samples were then taken from the mixture: one was applied to paper and dried, and the other was separated electrophoretically into nucleotides by the method of Markham and Smith [31]. The stains of the nucleotides were localized by means of the ultrachemiscope and the total activity of each nucleotide and of the original mixture was determined. All the stains were then eluted with 0.5N $HClO_4$ and the absorption of the original stain and the sum totals of the four separated nucleotides were determined. It was found that the values of the total activity of each of the four nucleotides were very close to each other (A-172, G-170, U-156, C-188 imp/min), which indicates a uniform incorporation of the label into the different nucleotides of the RNA. On the other hand, the ratio between the total activity of the four nucleotides to their absorption (1.1 imp/min/unit of extinction) was approximately equal to the ratio between the activity of the original solution and its absorption (1.2 imp/min/unit of extinction). Consequently the whole activity of the nucleotide fraction depended upon the RNA nucleotides.

Similar experiments with the same results were carried out also on high-polymer RNA, in which no carrier was added. Thus the results of the trial showed that the method used for purification of the RNA was perfectly satisfactory.

ANALYTICAL METHODS

In order to calculate the quantitative ratio between all the various fractions, the following quantitative determination was made. The unit for the calculation was taken to be the quantity of RNA. The following ratios were determined: a) total RNA:DNA; b) C-RNA-HP : C-RNA-LP : C-RNA-NE; c) NS-RNA-HP : N-RNA-E ; N-RNA-NE: DNA. Ratio (a) enabled the total amount of RNA in the cell to be judged; ratio (c) gave the content of NS-RNA-HP, N-RNA-E and N-RNA-NE; the quantity of cytoplasmic RNA could be calculated by the difference between (a) and (c), and then from ratio (b) the content of C-RNA-HP, C-RNA-LP and C-RNA-NA could be calculated. The separation of DNA and RNA was achieved by the method of Schmidt and Tannhauser, and their quantitative determination by the methods of Meibaum [32] and Dische [33] and spectrophotometrically.

EXPERIMENTAL RESULTS AND DISCUSSION

In the table we present the results of the incorporation of P^{32} per hour in the various RNA fractions, obtained in two experiments using different methods of fractionation (method II, column 1, and method III, column 2). These results confirm our previous experimental findings [18,19], and moreover the difference between the incorporation into the various fractions was more marked than that described earlier. The value of ratio RA N-NRA-NE : RA C-RNA-HP was 220-240. Attention should be directed to the relatively high incorporation into N-RNA-E, NS-RNA-HP and C-RNA-NE, although this was much less than the incorporation into N-RNA-NE. In column 3 we show the percentages of the total RNA pertaining to its fractions, obtained by quantitative analysis of the balance of RNA of the same cells. By multiplying the RA by the content of a given fraction, the total activity

of the fraction could be calculated and the proportion of the total activity of the RNA of the cell due to the particular fraction deduced (column 4). It will be seen from the table that in the early stages of incubation with the N-RNA-NE fraction, accounting for only about 6% of the total RNA of the cell (in this experiment), 72.4% of the total radioactivity incorporated into the RNA was combined. Only about 3.3% of the total activity was present in the C-RNA-HP, forming 66% of the total RNA of the cell. Such relative proportions indicate the possible role of N-RNA-NE as a precursor of the other fractions of the cell RNA.

TABLE. Incorporation of P^{32} in Vitro into RNA Fractions of Ehrlich's Ascites Carcinoma Cells (Incubation for 1 Hour)

Fraction	Method of fractionation			
	II	III		
	RA of fraction*	RA of fraction	Content of fraction in relation to total RNA, %	Proportion of total RNA activity due to fraction, %
N-RNA-NE	89	55	6	72.4
N-RNA-E	7.2	10	4	8.8
NS-RNA-HP	0.4	3.2	8	5.6
C-RNA-HP		0.23	66	3.3
C-RNA-NE		4.0	6	5.3
C-RNA-LP	4.7	2.1	10	4.6

*$\overline{RA}$ of the fractions is expressed in imp/min/μg RNA.

In order to study the role of N-NRA-NE and the relationships between the various RNA fractions, we investigated the incorporation of P^{32} at different intervals of time during incubation in vitro (Fig. 1). This investigation showed, however, that throughout the entire period (from 10 min to 4 hr) the RA of all the fractions rose, including that of the N-RNA-NE, so that in none of the RNA fractions was a state of equilibrium reached. As performed in this way, therefore, this experiment gave little information on the analysis of the relationships between the different RNA fractions. The presence of a latent period for the incorporation of P^{32} into the HP-RNA was all that could be observed.

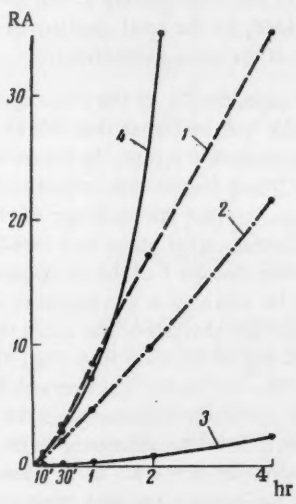


Fig. 1: Incorporation of P^{32} into the RNA fractions during incubation of Ehrlich's carcinoma cells and ascites fluid in vitro. 1) N-RNA-NE (RA of fraction reduced tenfold); 2) RNA-LP; 3) RNA-HP; 4) N-RNA-E. RA—in imp/min/μg RNA.

In subsequent experiments we therefore studied the change in the activity of RNA during the incubation in vivo of cells, preliminarily labeled with P^{32} in vitro for one hour, with unlabeled P. Such cells remained completely viable. The absorption of nonradioactive P by the cells led to a rapid fall in the RA of the free nucleotides, as a result of which after four hours the growth of the RA of the total RNA had practically ceased (Fig. 2). From this analysis of the curve of the change in the RA of the different RNA fractions it was possible to form definite conclusions regarding their relationships with each other and the possibility of their synthesis from free nucleotides. In Figs. 3 and 4 are shown the curves of the change in the RA of the RNA fractions during incubation for 16-18 hours in vivo with unlabeled P. In two experiments, one of which is illustrated in Fig. 3, fractionation was carried out by method II, and in two others (Fig. 4, a, b) by method 3.

The principal indicators in the analysis of the results obtained were as follows: 1) the distribution of the total activity of the RNA between its fractions in the early periods of incubation and its redistribution in later periods; 2) the relationship between the rate of change of the RA of a fraction and the RA of

precursor: nucleotides or other RNA fractions; 3) the localization of the point of the maximum on the curve of change of the RA.

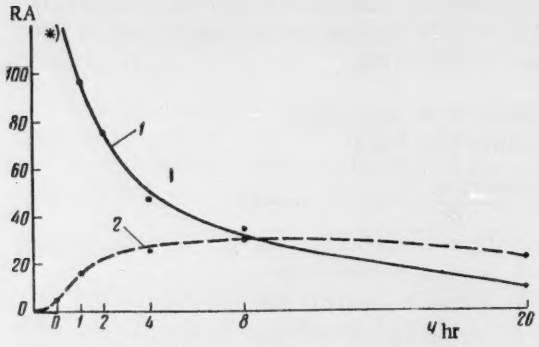


Fig. 2. Change in the RA of the free nucleotieds (1) and total RNA (2) during incubation of the cells. The cells were incubated 1 hour with P^{32} in vitro (here and in the subsequent figures shown by a blackened section on the axis of abscissas) and then with nonradioactive phosphate in vivo. The RA of the nucleotides at zero time was 150 imp/min.

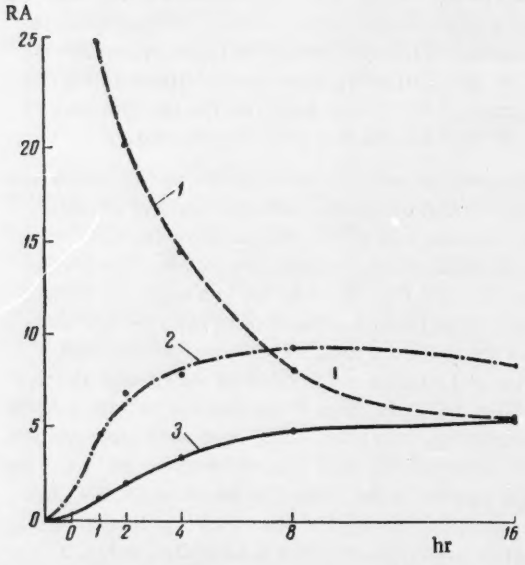


Fig. 3. Change in the RA of the various RNA fractions during incubation of cells. The cells were incubated for one hour with P^{32} in vitro and then with nonradioactive phosphate in vivo. Fractionation by method II. 1) N-RNA-NE; 2) RNA-LP; 3) RNA-HP.

We have already discussed the distribution of the total activity between the RNA fractions in the early periods. At the end of incubation the RA of the various fractions of the RNA was almost identical, that of the N-RNA-NE being the lowest. Thus the total activity due to this particular fraction at this period correspond approximately to the percentage content of the fraction, and the greater part of the P^{32} was combined with the C-RNA-HP. These results, however, give only indirect evidence of the role of N-NRA-NE as a precursor of certain other forms of cell RNA. More valuable information is given by the analysis of two other factors, which is based on the assumption given below.

Investigations in recent years have shown that the precursors of RNA are free nucleotides in the form of nucleoside di- or triphosphates [34, 35]. We therefore determined the RA of the phosphorus of the acid-soluble nucleotide fraction, considering that its phosphorus was the precursor of the RNA phosphorus. This assumption has two limitations. In the first place, the β- and γ- P atoms in the molecule of nucleoside polyphosphate are metabolized more quickly than the α-atom, so that with a fall in the RA of the P_{inorg}, their RA will fall more rapidly. Meanwhile it is the α-atom of the nucleotide that is incorporated into the RNA. On the other hand, the RA of the P-UMP, P-CMP and P-GMP will probably fall more rapidly at this period than the RA of the P-AMP, for the total quantity of AMP is greater than that of the other nucleotides.

In the first case, the RA of the P incorporated into the nucleic acids will be higher than the RA of the whole P of the nucleotide fraction. In the second case it will be lower. These factors thus cancel each other out to some extent. Further the exchange of phosphorus between the nucleotides takes place very rapidly. It is thus highly probable that the P of the nucleotide fraction investigated may be regarded as the precursor of the P-RNA. In fact, from the analysis of the curve of the RA of the nucleotides and of the total RNA (Fig. 3), it follows that the P of the nucleotide fraction satisfies the requirements of a precursor: the change in the RA of the RNA is proportional to the difference in RA of the nucleotides and RNA ($db/dt = v(a - b)/B$, where a and b are the RA of the nucleotides and RNA respectively, B is the quantity of RNA; v the velocity of its synthesis) [36].

The point of maximum RA of the total RNA approximately coincides with the point of intersection of the curves of the RA of RNA and nucleotides (at $db/dt = 0$; $a = b$). Further, in the analysis of the movement of RNA from one fraction to another, we start from two assumptions: 1) the transfer of RNA from one fraction to

another (from some structures to others) is irreversible; 2) the fractions of RNA are homogeneous. The first assumption is based on the fact that the high-polymer RNA in the composition of the RNP granules enters into complexly organized, differentiated cell structures. This makes it unlikely that states of equilibrium can exist between the RNA of the different fractions.

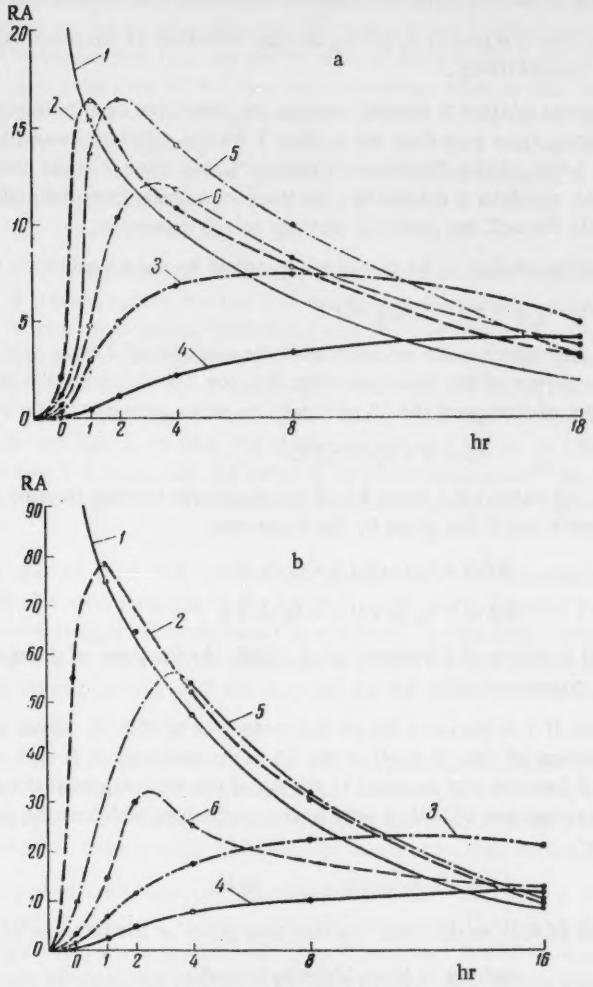


Fig. 4. Change in the RA of the various RNA fractions during incubation of cells. The cells were incubated for one hour with P^{32} in vitro and then with nonradioactive phosphate in vivo. a and b: 1) nucleotides; 2) N-RNA-NE; 3) C-RNA-LP; 4) C-RNA-HP; 5) N-RNA-E; 6) C-RNA-NE (a) and NS-RNA-HP (b).

The second assumption concerning the metabolic homogeneity of the RNA fraction is much more limited in its character in view of the complexity of the internal structure of the cell and the diversity of the structures into the composition of which enter the ribonucleoprotein granules and their fractions. Since the heterogeneity of the RNA fraction primarily affects the localization of the point of maximum RA of the fraction, the limitation of this assumption decreases the value of the conclusions obtained from the analysis of the position of the maximum RA of the fractions.

The limitations of these assumptions, described above, together with the inadequate definition of the precursor of RNA in the experiments with P^{32}, permit only a qualitative analysis of the curves to be made, based on the general requirements of the precursor and the product. Nevertheless it is desirable to present a few equations which may be useful both for the analysis of the present experimental findings and also for future investigations.

If we accept the above-mentioned assumptions, we may describe the following scheme of conversions: $A \xrightarrow[v_1]{} B \xrightarrow[v_2]{} C$, where A represents nucleotides, B the RNA fraction synthesized primarily; and C the RNA fraction into which the RNA is transferred from fraction B; v_1 and v_2 are the velocities of the reactions; the values of the RA of A, B and C are a, b and c respectively.

Let us assume that an interval of time T elapses between the formation of a molecule of RNA-B and its transition to RNA-C. Two limiting cases may then arise: when T for the different molecules differs considerably (in this case the B fraction is a homogeneous "metabolic cauldron"), and when T is the same for all the molecules of RNA-B. The latter state of affairs is reasonable, for the biosynthesis, transport and conversion of the ribonucleoprotein granules within the cell are probably strictly orderly processes.

In the general equation of the change in RA B may be described by the equation

$$db/dt = v_1(a - b)/B - v_2(x-b)/B$$

where $v_1(a-b)/B$ characterized the change in RA on account of the synthesis of B from A; $v_2(x-b)/B$ the change in RA on account of the transfer of part of the molecules from fraction B to C (where x is the RA of the molecules leaving fraction B). The velocity of change of the RA of C will be given correspondingly by

$$dc/dt = v_2(x-c)/C.$$

In the first case (if T has any value) the mean RA of the molecules leaving fraction B is equal to b and the changes in the RA of fractions B and C are given by the equations:

$$db/dt = v_1(a-b)/B = k_1(a-b); \quad (1)$$

$$dc/dt = v_2(b-c)/C = k_2(b-c), \quad (2)$$

which is identical with the usual equation of Zilversmit et al. [36]. At the point of the maximum, when $db/dt = 0$ or $dc/dt = 0$, then $a = b$ or, correspondingly, $b = c$.

Different relationships arise if T is the same for all the molecules of RNA-B. Since the RA of the RNA molecule synthesized at any moment of time is equal to the RA of the nucleotides at that moment, the RA of the RNA molecules leaving the B fraction will be equal to the RA of the nucleotides at time T away from the given moment (a_T), when those molecules of RNA-B were being synthesized which at the present moment are passing into the C fraction ($x=a_T$).

Hence: $$db/dt = v_1(a-b)/B - v_2(a_T-b)/B.$$

If the synthesis and transfer of RNA to the other fraction take place at the same velocity ($v_1 = v_2$), then

$$db/dt = v_1(a-a_T)/B = k_1(a-a_T). \quad (3)$$

For those periods of incubation at which $a_T = 0$,

$$db/dt = v_1 \cdot a/B = k_1 \cdot a. \quad (4)$$

Correspondingly

$$dc/dt = v_2(a_T-c)/C = k_2(a_T-c) \quad (5)$$

and for early periods of incubation $dc/dt = 0$; the latent period for C will be exactly equal to the time interval T. At the point of maximum for b: $a = a_T$ and for c: $c = a_T$, and moreover at the point of maximum $b > a$ and $c > b$. If the course of the process follows the second variant, T may be easily determined directly from analysis of the graph of the change in RA.

It is evident that, in fact, T may vary considerably and the curves may be described by an equation lying intermediately between the first and second cases.

In the analysis of the curves of incorporation of P^{32} obtained in our experiments the following questions arise. What fractions of RNA may be synthesized from free nucleotides? For this, they must satisfy equations 1, 3 or 4. In other words, since the RA of the nucleotides is maximum in the interval 0-1 hour, at this period the rate of increase in the RA of the fraction being synthesized from free nucleotides must also be considerably higher than at subsequent intervals of time.

Of all the fractions studied, these conditions are satisfied only by N-RNA-NE and, partially, by N-RNA-E and RNA-LP. The increase in the RA of C-RNA-HP, NS-RNA-HP and C-RNA-NE is maximal in the interval 1-2 hours, when the RA of the nucleotides falls sharply by comparison with the interval 0-1 hour. Conversely, the RA of N-RNA-NE rises during the interval 0-1 hour with maximum velocity and to a maximum level, comparable to the RA of the nucleotides, after which it falls parallel with the fall in the RA of the nucleotides. The RA of RNA-LP rises more intensively in the interval 0-1 than in the interval 1-2 hours, although not sufficiently to explain fully the increase in its RA on account of synthesis from free nucleotides. It is evident that N-RNA-E may be synthesized from free nucleotides, although the course of the curve of its RA may be more simply explained on the assumption that this if formed from the N-RNA-NE fraction.

Since the RNA-HP of the cytoplasm, forming the greater part of the RNA of the cell, is not synthesized from free nucleotides, in the analysis of the results a second question arises: what fraction of the RNA may be the precursor of RNA-HP? Barnum and Huseby considered that the precursor of the RNA of the micorsomes (i. e. C-RNA-HP) is the RNA of the supernatant fraction (i.e. C-RNA-LP). Our results, however, show that no proportionate relationship exists between the growth of the RA of C-RNA-HP and the difference in the RA of RNA-LP and RNA-HP (equation 2). This is espeically obvious from a comparison between the RA of RNA-HP and RNA-LP in the interval 1-2, 2-4 and 4-8 hours. In fact, the difference between the RA of RNA-LP and the RA of RNA-HP is highest in the period 2-4 or 4-8 hours, and the velocity of incorporation of P^{32} into RNA-HP at this time falls considerably. A discrepancy arises also if an attempt is made to apply equation 5 to the change in the RA of RNA-HP and RNA-LP.

The synthesis of RNA-HP from RNA-LP could be described from the standpoint of equation 5. In order to account on this basis for the maximum rise in the RA of RNA-HP in the interval 1-2 hours, it would have to be assumed that T for RNA-LP is approximately equal to one hour. In this case, however, the curve of the RA of RNA-LP would have to reach its maximum much faster (between the first and second hour at $a = a_T$), and almost immediately after its intersection with the curve of the RA of the nucleotides it would have to start to fall rapidly.

Thus C-RNA-LP is not the precursor of C-RNA-HP. The fractions C-RNA-NE, N-RNA-NE and N-RNA-E, from the shape of the curve of change in RA, may all act as precursor of C-RNA-HP (equation 2). Of these, however, as shown above, only N-RNA-NE and N-RNA-E may be synthesized from free nucleotides, and C-RNA-NE is evidently fromed on account of the transfer of RNA from another fraction, probably from N-RNA-NE.

Thus the primary precursors of RNA-HP are evidently N-RNA-NE and N-RNA-E. Since in the early periods nearly all the radioactivity is localized in the N-RNA-NE, it is most likely that this is the newly formed high-polymer RNA of the ribonucleoprotein granules of the cell, and that N-RNA-E, together with NS-RNA-HP and C-RNA-NE lie on the path of transition of N-RNA-NE into C-RNA-HP. In any case, irrespective of whether N-NR RNA-E is formed from N-RNA-NE or from free nucleotides, the biosynthesis of the high-polymer RNA of the cell is associated with the activity of the chromosome-nucleolar apparatus, which contains both N-RNA-NE and N-RNA-E.

So far as the localization of the maxima of the curves of the RA of the various fractions is concerned, we have already remarked that their importance is very limited. Nevertheless it should be mentioned that the position of the maximum RA of the N-RNA-NE on the curve of the RA of the nucleotides corresponds to the synthesis of N-RNA-NE taking place directly from nucleotides. The same considerations apply to the situation of the maximum RA of the C-RNA-NE and N-RNA-E on the curve of the RA of the N-RNA-NE or above it. The localization of the maximum for the NS-RNA-HP, like the whole course of the curve, clearly demonstrates the heterogeneity of this fraction. The reason for this is evidently that besides RNA of the nuclear sap, it also contains the RNA of the cytoplasmic contaminants.

It may thus be concluded from our findings that the biosynthesis of high-polymer RNA takes place in the chromosome-nucleolar apparatus, and that the newly formed RNA evidently is the RNA of the N-RNA-NE fraction. It is highly probable that N-RNA-E, NS-RNA - HP and C-RNA-NE are intermediate fractions along the path N-RNA-NE → C-RNA-HP, although final conclusions will have to await the obtaining of adequate data, for the characteristics of these three RNA fractions are insufficiently known.

Finally, the third and difficult question arising in the analysis of the experimental results, concerning the origin of the RNA-LP, contains much that is unexplained. As we have stated above, the course of the curve of its RA (the maximal growth in the RA in the interval 0-1 hour) may partially be explained by exchange with the P of the free nucleotide fraction, probably on account of the terminal attachment of the latter [37]. However, the velocity of growth of its RA in the period 1-2 hours falls only a little, in spite of the sharp fall in the RA of the nucleotides. This is probably associated with the fact that the formation of new RNA-NP also does not take place in the cytoplasm, but is associated with the activity of the nucleus.* In fact, as our experiments have shown [38], in the liver of the rat the incorporation of P^{32} into the low-polymer RNA of the nuclear sap is much greater than that into the cytoplasmic RNA.

A more accurate picture of the interconnections between the RNA fractions of the cell structures must await, in the first place, further information on the RNA of these fractions and, in particular, of the nuclear RNA. Secondly, it is necessary to use more convenient precursors in experiments on biosynthesis, so that it will be possible to make not merely a qualitative, but also a quantitative application of the equations which we have described to the curves of the change in the RA of the RNA. Work in this direction is proceeding at the present time.

SUMMARY

Incorporation of P^{32} into the free nucleotides and different RNA fractions was studied in Ehrlich ascites carcinoma cells. The cells were incubated one hour with P^{32} in vitro and further with non-radioactive phosphate 1-20 hours in vivo.

After 1 hour incubation the nuclear RNA-N which constitutes only 6% of total cell RNA, contained about 72% of total radioactivity of RNA, whereas the cytoplasmic high-polymer RNA contained only about 3% of radioactivity. After 16-18 hours in vivo incubation the main part of P^{32} was found in the latter fraction.

The analysis of the RNA specific activity curves shows that the high-polymer RNA of the cell is synthesized in the nucleolochromosomal apparatus and transferred into the cytoplasm. The RNA synthesized de novo from free nucleotides is localized in the nuclear RNA-N fraction. Nuclear RNA-E, high-polymer RNA of the nuclear sap and cytoplasmic non-extractable RNA were supposed to play the role of intermediate on the way nuclear RNA-N→ →cytoplasmic high-polymer RNA.

The incorporation of P^{32} into the low-polymer RNA probably depends on its synthesis in the nucleus and on the exchange reaction with free nucleotides.

The authors express their gratitude to Professor I. B. Zbarskii for his valuable advice in the course of this work.

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*In the experiment shown in Fig. 3, in which we investigated the activity of the total RNA-LP and not of the C-RNA-LP, the growth of its RA in the interval 0-1 hour was much more intensive than its growth in the subsequent intervals, by comparison with the experiments in Fig. 4, a, b.

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ACCELERATION OF THE EXCRETION OF YTTRIUM, CERIUM AND LEAD FROM THE BODY WITH THE AID OF ETHYLENE-DIAMINE-TETRAACETIC ACID, DIETHYLENETRIAMINE-PENTAACETIC ACID AND THE DIAMINODIETHYL ESTER OF TETRAACETIC ACID

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The ability of certain organic substances and, in particular, the complexons-derivatives of iminodiacetic acid – to form highly stable, readily soluble intra-complex compounds with cations of metals not only in vitro but also within the body has been widely used in recent years in the treatment of poisoning with radioactive isotopes and toxic heavy metals [1,7]. A complexon now well known in therapeutics is $Na_2CaEDTA$, which, when injected intravenously, causes a sharp rise in the excretion of manganese, zinc, yttrium, lanthanum, cerium, lead, plutonium etc. in the urine [1,2,7].

Subsequent research in the field of complexon therapy has been directed to the search for new and more effective preparations, and also to the discovery of a possible selective action in relation to a given metal or group of metals. In this direction we have studied about 30 complex-forming substances from the group of phosphates and iminopolycarboxylic derivatives [1,3]. All with the exception of uramildiacetate and 1,2-dimethylethylenediamine tetraacetate were much less effective in promoting excretion of metals from the body than EDTA. Admittedly hexametaphosphate showed specific activity in relation to radioactive cerium and plutonium, by increasing to a greater degree than did EDTA the elimination of these metals in the urine, but the marked toxicity of the preparation prevented its use for therapeutic purposes being recommended.

Recently [8-12] new complexons have been tested – diethylenetriamine pentaacetic acid (DPTA) and diaminodiethyl ester of tetraacetic acid (DDTE) – and these have been found to be much more effective than EDTA in preventing the deposition of Y, Ce and Pb in the tissues and in accelerating their excretion from the body.

These findings are of great theoretical and practical interest. We accordingly carried out experiments to determine the efficacy of these two new complexons and of EDTA for purposes of comparison in the elimination of both Y and Ce, as already studied [9-11], and also of one of the most toxic of the metals – lead – from the body.

EXPERIMENTAL METHOD

As experimental animals we used male white laboratory rats. Solutions of microquantities of yttrium ($Y^{91}Cl_3$), cerium ($Ce^{144}Cl_3$) and lead ($Pb^{210}(NO_3)_2$) were injected without carrier into the dissected caudal vein in a dose of 3-5 μC to each rat (solution pH 2; volume 0.3-0.5 ml). The efficacy of the calcium salts of the complexons $CaNa_2EDTA$, $CaNa_2DDTE$ and $CaNa_3DTPA$ in solutions of pH 7.3 was tested at both early and late

periods after poisoning. In the first case, 2-3 minutes after injection of the metal, 20 μmole of the corresponding complexon in a volume of 0.5 ml was injected into the other caudal vein; the animals were kept for 24 hours in metabolism cages so that their urine and feces could be collected separately, after which they were sacrificed and the concentration of the metal in the organs and tissues and in the excreta during the first 24 hours was determined. In the second case the animals were kept in metabolism cages for 16 days after injection of the metals, and on the eighth and 13th days they received intraperitoneal injections of 100 μmole of the corresponding complexons in a volume of 1 ml. In the experiments with Ce the rats were then sacrificed for the purpose of determining the total balance of radioactivity contained in the tissues and excreta. Since a satisfactory balance was obtained in thes experiment, amounting to an average value of 99.309 ± 0.866% of the quantity of radioactive isotope injected into each rat, in the subsequent experiments (with Y and Pb) the animals were not sacrificed, but after an inverval of 18 days they were again placed in the metabolism cages (from the 35th to the 46th day), and on the 38th and 43rd days they again received injections of 100 μmole each of the corresponding complexons. The rats were then removed from the experiment and used for other purposes. Thus the efficacy of the complexons when administered late in the experiments with yttrium and lead was determined only by their influence on the excretion of the metals in the urine and feces.

In order to determine the content of radioactive isotopes, the internal organs and the feces were dried for 5 hours at T 150°; the femur and the rest of the cadaver were incinerated in a muffle furnace (in the case of Y and Ce at 600°, in the case of Pb at 250°); the urine together with the EDTA solution with which the metabolism cages were washed out daily were evaporated down to a volume of 20-80 ml. Multiple specimens were prepared from the internal organs, the cadaver, the femur and the feces, all of which were ground to powder in a special apparatus, and also from the urine solution, on standard aluminum targets for measurement of their content of radioactivity by means of the B-2 radiometer. The samples containing Pb^{210} were kept for 35 days, and on the attainment of radioactive equilibrium, were measured under the radiometer as the daughter product, Bi^{210}. To determine the quantity of radioactivity injected into the animals, multiple samples were prepared both from the solution injected and from the whole ashes of 2-3 rats in each experiment, the animals being sacrificed immediately after receiving an injection of the corresponding source of radiation.

The content of isotopes in the skeleton was calculated in the usual way by multiplying the radioactivity measured in the femur by 20. The content of isotopes in the soft tissues was the difference between the radioactivity of the whole cadaver (except the liver and kidneys) and the radioactivity of the skeleton.

The results showing the content of Pb and Y in the urine and feces of the rats after late administration of complexons were expressed as percentages of the radioactivity injected; in those cases when the isotope content of both the excreta and the tissues was measured, the results were shown as percentages of the balance, i. e. as percentages of the total radioactivity discovered. If the experiment was carried out carefully, the value of the balance was very close to the value of the radioactivity injected, and thus was an important index of the radioactivity. In the tables we therefore show the balance in individual experiments, expressed as percentages of the radioactivity injected.

EXPERIMENTAL RESULTS

The simutaneous performance of experiments with EDTA, DDTE and DTPA by the same technique and on a sufficiently uniform material enables the efficacy of these preparations to be compared in relation both to each individual metal and to different metals.

The early administration of complexons. The data that are given in Table 1 indicate that DDTE and DTPA caused a sharp fall in the deposition of Y in the tissues and increased its excretion in the urine (the differences between the values of excretion of Y in the feces are not statistically significant). They were more effective in this direction than EDTA; only in the degree of deposition of Y in the soft tissues were the differences not statistically significant ($P_{EDTA-DDTE} = 0.98$; $P_{EDTA-DTPA} = 0.23$). The differences in the efficacy of the two new complexons consisted essentially of a more marked decrease in the deposition of Y in the liver under the influence of DTPA and an increase in its excretion in the urine (the differences are statistically significant). A greater fall in the deposition of the isotope in the skeleton was also observed under the influence of DTPA than with DDTE (from 70.32 to 1.08 and to 5.02% respectively), although because of the considerable error of the mean in the latter case (±1.620) this difference is not statistically significant ($p = 0.07$), assuming that the value of p, the usually accepted limit of reliability, is equal to 0.05.

TABLE 1. Content of Yttrium (%) in the Tissues and Excreta of Rats 24 Hours After Intravenous Injection of the Isotope and Complexons (Complexons injected in doses of 20 μM 2-3 minutes after injection of the isotope. Balance equal to 97.177±1.376% of quantity of Y injected. Three animals tested in each group)

Tissues and excreta	Control	EDTA	DDTE	DTPA
Liver	4.59±0.193	1.71±0.081	0.84±0.024	0.45±0.040
Kidneys	2.24±0.240	1.02±0.023	0.55±0.023	0.43±0.049
Skeleton	70.32±4.550	15.00±0.704	5.02±1.620	1.08±0.182
Soft tissues	10.80±2.530	3.30±0.351	3.33±1.421	2.65±0.299
Urine	10.24±1.157	75.97±2.053	88.20±0.821	93.44±0.259
Feces	1.81±1.157	3.00±0.362	2.06±1.185	1.95±0.321
Weight of rats, g	157±3.85	159±2.33	162±5.50	158±1.87

TABLE 2. Content of Cerium in the Tissues and Excreta of Rats 24 Hours After Intravenous Injection of the Isotope and Complexons. (Complexons injected in doses of 20 μM 2-3 minutes after injection of the isotope. Balance equal to 96.112±0.823% of quantity of Ce injected)

Tissues and excreta	Control	EDTA	DDTE	DTPA
Liver	50.05±2.640	17.16±1.120	9.48±1.428	5.22±0.987
Kidneys	2.98±0.256	1.02±0.082	0.60±0.030	0.56±0.093
Skeleton	34.27±2.770	36.20±0.403	20.47±1.945	3.67±1.045
Soft tissues	10.48±0.955	9.83±1.101	3.48±0.968	6.57±2.410
Urine	1.59±0.032	34.37±1.504	62.29±2.292	81.02±2.667
Feces	0.63±0.095	1.42±0.091	3.68±1.161	2.96±0.215
Weight of rats, g	169±4.83	171±3.78	166±5.13	173±5.68

The figures in Table 2 show that DDTE and DTPA diminish to a greater degree than EDTA the deposition of cerium in the tissues (only the differences in deposition of the isotope for the soft tissues in the "control" and "DTPA" groups are not statistically significant; $p = 0.2$) and they increase its excretion in the urine. The differences between these complexons are especially marked in the figures relating to the skeleton: whereas EDTA may possibly ($p = 0.52$) even slightly increase the deposition of Ce in bone tissue, DDTE and, in particular, DTPA reduced its deposition from 34.27 to 20.47 and to 3.67% respectively; the difference in efficacy between DDTE and DTPA in this case is also statistically significant ($p = 0.0016$). Besides the considerable differences in the quantities of Ce excreted in the urine under the influence of the various complexons, the increased excretion of the isotope in the feces under the influence of EDTA ($p = 0.004$) and DTPA ($p = 0.00055$) should be mentioned, DTPA being more effective in this case ($p = 0.0027$); the difference between the "control" and "DDTE" groups was not statistically significant ($p = 0.125$).

The results of the experiment with lead are shown in Table 3, and they indicate that DDTE was less effective than EDTA; DDTE had a less pronounced effect than EDTA in diminishing the deposition of lead in the kidneys and skeleton, but it also increased its elimination in the urine (differences statistically significant). At the same time DTPA caused a greater decrease in the deposition of Pb in the skeleton and an increase in its excretion in the urine than did EDTA. The difference between DDTE and DTPA in lowering the deposition of Pb in the liver is also significant. Finally, of all the complexons, only DTPA led to a statistically significant decrease in the deposition of Pb in the soft tissues.

TABLE 3. Content of Lead in the Tissues and Excreta of Rats 24 Hours After the Intravenous Injection of the Isotope and Complexons. (Complexons injected in doses of 20 μM 2-3 minutes after injection of the isotope. Balance equal to 97.00±1.151% of quantity of Pb injected)

Tissue and excreta	Control	EDTA	DDTE	DTPA
Liver	5.08±0.353	2.25±0.382	3.40±0.505	1.66±0.110
Kidneys	7.60±0.338	2.05±0.301	3.33±0.214	1.73±0.094
Skeleton	44.17±3.310	14.02±0.854	34.35±0.840	6.73±0.381
Soft tissues	24.56±2.383	18.42±3.303	16.72±2.075	10.99±2.655
Urine	13.38±0.797	58.38±3.363	38.69±1.185	73.43±3.150
Feces	4.91±1.202	4.88±0.183	3.51±1.897	5.46±0.247
Weight of rats, g	123±3.05	133±2.51	120±9.86	125±9.45

The late administration of complexons. The data that are given in Table 4 indicate that the administration of complexons in later stages of the experiment led to the increased elimination of yttrium from the body.

TABLE 4. Excretion of Yttrium in the Urine and Feces of Rats Receiving 100 μmole of Complexons Given Intraperitoneally on the 8th, 13th, 38th, and 43rd Days After Injection of the Isotope. (Two rats were investigated in each group)

Days	EDTA		DDTE		DTPA	
	Urine	Feces	Urine	Feces	Urine	Feces
1	10.60±1.032	2.11±0.679	10.82±0.194	1.53±0.010	10.39±0.131	1.19±0.140
2	1.07±0.079	0.85±0.546	1.14±0.064	0.63±0.022	1.01±0.063	0.90±0.232
3	0.69±0.146	0.97±0.126	0.76±0.015	0.83±0.077	0.67±0.043	0.64±0.017
4	0.58±0.150	0.94±0.070	0.52±0.028	0.91±0.300	0.58±0.042	1.03±0.067
5	0.37±0.015	0.79±0.186	0.40±0.057	1.11±0.268	0.42±0.020	0.93±0.068
6	0.29±0.030	0.36±0.033	0.32±0.032	0.32±0.050	0.31±0.002	0.54±0.120
7	0.22±0.031	0.84±0.100	0.23±0.016	0.55±0.082	0.26±0.002	0.99±0.045
8 *	1.67±0.270	0.60±0.020	2.71±0.107	0.93±0.026	2.92±0.120	1.33±0.182
9	0.36±0.019	0.46±0.079	0.84±0.168	0.47±0.047	0.84±0.041	0.79±0.153
10	0.26±0.027	0.48±0.015	0.47±0.021	0.24±0.047	0.58±0.003	0.34±0.058
11	0.18±0.010	0.32±0.135	0.37±0.003	0.12±0.018	0.47±0.017	0.17±0.013
12	0.15±0.012	0.34±0.122	0.23±0.028	0.12±0.035	0.34±0.043	0.16±0.010
13 *	1.08±0.246	0.22±0.035	1.80±0.035	0.24±0.010	1.77±0.170	0.30±0.006
14	0.22±0.027	0.21±0.038	0.32±0.013	0.18±0.013	0.42±0.022	0.13±0.007
15	0.13±0.025	0.22±0.045	0.24±0.013	0.11±0.030	0.32±0.006	0.12±0.025
16	0.09±0.010	0.14±0.008	0.16±0.004	0.06±0.017	0.25±0.020	0.05±0.020
35 **	0.05±0.011	0.07±0.003	0.05±0.003	0.06±0.007	0.06±0.004	0.08±0.012
36	0.04±0.008	0.03±0.004	0.04±0.001	0.08±0.011	0.04±0.007	0.06±0.007
37	0.04±0.010	0.08±0.007	0.03±0.026	0.07±0.018	0.04±0.005	0.06±0.011
38 *	0.42±0.092	0.03±0.002	0.60±0.005	0.07±0.014	0.61±0.112	0.10±0.023
39	0.03±0.012	0.07±0.004	0.10±0.004	0.07±0.005	0.15±0.004	0.06±0.009
40	0.05±0.003	0.07±0.006	0.07±0.004	0.06±0.007	0.11±0.001	0.05±0.0005
41	0.04±0.000	0.04±0.003	0.05±0.001	0.03±0.001	0.10±0.001	0.04±0.008
42	0.03±0.001	0.03±0.013	0.04±0.004	0.05±0.005	0.07±0.000	0.06±0.005
43 *	0.46±0.055	0.03±0.004	0.55±0.053	0.07±0.010	0.50±0.066	0.03±0.0005
44	0.06±0.014	0.05±0.001	0.09±0.012	0.05±0.010	0.10±0.006	0.05±0.014
45	0.03±0.004	0.04±0.008	0.06±0.011	0.04±0.003	0.08±0.001	0.03±0.005
46	0.02±0.001	0.04±0.012	0.04±0.009	0.04±0.011	0.07±0.004	0.03±0.003
Weight of rats, g	189 & 244		192 & 243		181 & 237	

Note. Here and in Table 6: * -injection of complexons; ** -between the 17th and 35th days the animals were not kept in metabolism cages.

As when the complexons were administered early, DDTE and DTPA were more effective than EDTA. However, in contrast to the early administration, the late administration of DDTE and DTPA led to roughly equal increases in the excretion of Y in the first day after the injections; DTPA showed a slight advantage because on subsequent days the excretion of the isotope remained on a higher level, which ultimately gave a larger total excretion in the urine (by approximately 1%). A fact deserving particular attention was that previously reported by Catsch [11], that increased excretion of the isotope in the urine took place not only in the first, but also in subsequent days of administration of DTPA (in our experiments the increased excretion lasted five days or more, as seen in Table 4); DDTE had a similar but less prolonged action. We must also mention the increased excretion of Y in the feces during the first two periods of administration of DDTE and DTPA, which was not observed after administration of EDTA.

TABLE 5. Excretion of Cerium in the Urine and Feces of Rats Receiving 100 μmole of Complexons Intraperitoneally on the 8th and 13th Days After Injection of the Isotope Balance 99.309±0.866% of total cerium injected. Four rats in each group investigated

Days	EDTA		DDTE		DTPA	
	Urine	Feces	Urine	Feces	Urine	Feces
1	1.79±0.197	0.97±0.177	1.93±0.313	0.98±0.410	1.87±0.186	0.39±0.039
2	0.36±0.021	1.04±0.241	0.24±0.034	1.24±0.473	0.33±0.008	1.17±0.522
3	0.26±0.051	1.16±0.201	0.15±0.028	1.48±0.505	0.18±0.022	1.42±0.452
4	0.16±0.024	1.24±0.144	0.14±0.043	2.15±0.443	0.14±0.012	1.61±0.412
5	0.13±0.025	2.58±0.338	0.13±0.021	2.18±0.330	0.13±0.020	2.94±0.744
6	0.10±0.018	2.86±0.365	0.09±0.016	2.75±0.311	0.08±0.012	2.12±0.203
7	0.08±0.018	2.86±0.308	0.07±0.016	3.90±0.402	0.08±0.013	2.12±0.091
8	0.27±0.044	2.88±0.435	1.75±0.148	2.93±0.003	6.38±0.340	5.44±0.352
9	0.10±0.014	2.81±0.301	0.53±0.057	2.24±0.227	2.28±0.115	5.99±0.281
10	0.09±0.012	3.00±0.427	0.31±0.035	2.31±0.540	1.24±0.118	3.64±0.790
11	0.08±0.009	2.85±0.308	0.24±0.054	1.87±0.203	0.75±0.007	2.72±0.482
12	0.04±0.006	1.85±0.298	0.17±0.040	1.22±0.038	0.57±0.033	1.91±0.205
13	0.24±0.035	1.82±0.224	1.35±0.158	2.55±0.387	2.60±0.043	2.38±0.633
14	0.07±0.021	2.11±0.230	0.33±0.027	2.33±0.185	0.92±0.033	3.36±0.450
15	0.05±0.012	1.72±0.180	0.21±0.029	2.55±0.310	0.51±0.055	2.14±0.216
16	0.05±0.017	1.49±0.200	0.16±0.020	1.25±0.230	0.60±0.028	0.64±0.045
Weight of rats, g	166±5.44		168±4.30		171±7.36	

A similar pattern was observed during administration of complexons in the experiments with cerium (Table 5). In general, despite the relatively small doses of complexons (2 injections each of 100 μM on the eighth and 13th days after injection of isotope), the excretion of Ce in the urine over the period of 16 days reached a consierable magnitude, especially when DTPA was given. Estimation of the Ce content in the tissues of the animals in this series of experiments sacrificed on the 17th day gave the following results (mean figures are given; 4 animals were investigated in each group):

Organs, tissues	EDTA	DDTE	DTPA
Liver	21.00±0.755	20.52±2.450	5.40±1.771
Kidneys	0.71±0.080	0.48±0.066	0.45±0.013
Skeleton	28.87±2.550	26.93±1.168	25.88±1.381
Soft tissues	12.31±2.475	10.34±1.408	9.62±1.803

These figures show that DTPA mobilized the greater part of the isotope from the liver (EDTA-DTPA 0.0002; $P_{DDTE-DTPA} = 0.0035$). The considerable lowering of the content of Ce in the kidneys under the influence of DDTE ($p=0.067$) and DTPA ($p=0.026$) by comparison with EDTA was also statistically significant. With the use of these doses of complexons no statistically significant lowering of the cerium content in the skeleton was observed, which is in agreement with the results of Catsch's pilot experiment, but not with the results of his

main experiment [11], in which even with smaller doses of EDTA he obtained not merely a statistically significant, but a considerable mobilization of cerium from the skeleton (28.5% of the control).

TABLE 6. Excretion of Lead in the Urine and Feces of Rats Receiving Injections of 100 μmole of Complexons Intraperitoneally on the 8th, 13th, 38th, and 43rd Days After Injection of the Isotope. (Two rats in each group were investigated)

Days	EDTA		DDTE		DTPA	
	Urine	Feces	Urine	Feces	Urine	Feces
1	11,40±0,500	1,09±0,157	11,25±0,665	0,75±0,057	10,75±0,850	3,59±0,352
2	5,13±0,515	7,49±1,785	4,58±0,045	7,71±1,725	4,63±0,095	7,61±0,560
3	2,88±0,095	3,77±0,310	3,25±0,415	3,25±0,382	3,07±0,235	3,15±0,885
4	1,57±0,152	0,77±0,050	1,47±0,145	0,78±0,084	1,77±0,247	1,04±0,041
5	1,06±0,071	1,15±0,138	1,09±0,143	0,83±0,194	1,10±0,123	1,43±0,380
6	0,94±0,028	1,25±0,155	0,95±0,100	1,02±0,255	1,03±0,131	0,77±0,206
7	0,77±0,001	0,53±0,063	0,76±0,064	0,55±0,055	0,85±0,050	0,80±0,233
8*	1,92±0,135	0,61±0,095	0,76±0,163	0,55±0,119	2,23±0,206	0,64±0,001
9	0,57±0,063	0,18±0,086	0,43±0,036	0,36±0,037	0,52±0,084	0,29±0,026
10	0,39±0,043	0,33±0,062	0,33±0,073	0,36±0,051	0,39±0,025	0,50±0,093
11	0,26±0,043	0,20±0,019	0,26±0,050	0,26±0,055	0,27±0,018	0,25±0,030
12	0,21±0,005	0,15±0,013	0,23±0,020	0,17±0,010	0,21±0,019	0,16±0,040
13	1,06±0,140	0,17±0,037	0,27±0,038	0,18±0,065	1,10±0,074	0,26±0,066
14	0,20±0,017	0,11±0,038	0,15±0,022	0,05±0,004	0,18±0,016	0,19±0,077
15	0,19±0	0,18±0,012	0,15±0,020	0,18±0,030	0,17±0,003	0,13±0,043
16	0,16±0,003	0,12±0,002	0,16±0,024	0,13±0,015	0,19±0,048	0,15±0,091
35**	0,07±0,003	0,07±0,002	0,08±0,008	0,06±0,010	0,08±0,019	0,06±0,016
36	0,13±0,017	0,05±0,003	0,12±0,007	0,05±0,030	0,10±0,001	0,06±0,007
37	0,08±0,018	0,04±0,013	0,08±0,014	0,03±0,010	0,07±0,006	0,04±0,007
38*	0,47±0,172	0,07±0,021	0,15±0,011	0,10±0,019	0,45±0,044	0,09±0,047
39	0,08±0,003	0,06±0,027	0,06±0,004	0,08±0,020	0,05±0,004	0,08±0,006
40	0,11±0,010	0,07±0,007	0,11±0,017	0,08±0,018	0,09±0,016	0,07±0,016
41	0,10±0,006	0,05±0,002	0,08±0,009	0,10±0,012	0,09±0,015	0,05±0,016
42	0,08±0,021	0,05±0,015	0,08±0,004	0,04±0,002	0,10±0,006	0,06±0,025
43*	0,43±0,063	0,05±0,003	0,15±0,021	0,04±0,012	0,36±0,087	0,04±0,029
44	0,10±0,006	0,06±0,008	0,08±0,001	0,05±0,003	0,07±0,015	0,03±0,009
45	0,07±0,024	0,06±0,010	0,07±0,017	0,08±0,006	0,06±0,005	0,07±0,010
46	0,07±0,003	0,06±0,007	0,06±0,004	0,08±0,028	0,07±0,014	0,07±0,008
Weight of rats, g	138 & 166		145 & 160		146 & 159	

A somewhat different picture was observed in the experiments with lead (Table 6), in which DDTE had practically no effect when administered late. When administered early it gave the least marked effect. DTPA and EDTA increased the excretion of lead in the urine about equally, and had no effect on its excretion in the feces.

DISCUSSION OF THE RESULTS

Although they are of no essential practical interest, the experiments on early administration of complexons are very important not only as being the most convenient method of obtaining a fundamental solution of the problem of the efficacy of a given complexon, but also for the elucidation of a series of important theoretical problems.

It was on the basis of such experiments that in 1952 we postulated the hypothesis * of the interdependence between the constants of supplanting of calcium (K_{sup}) and the efficacy of the complexons in the elimination of metals from the body. According to this hypothesis, the higher the constant of stability of the complex of the given complexon with the metal (K_{me}) by comparison with that of its complex with calcium (K_{Ca}), i.e., the greater the ratio K_{me}/K_{Ca}, then the greater will be the effect of this complexon on the behavior of the metal

* This hypothesis and the developments leading from it were not published at that time, but were cited in the monograph by Balabukha and Fradkin [4]. (Unfortunately during the reprinting of the table showing the values of K_{sup} for the various metals in this monograph some mistakes appeared: the negative signs are absent before the figures in the columns Ba^{++} and Sr^{++}; in the same columns, instead of 2.8 and 1.9 the figures 22.8 and 1.0 respectively are given). In 1955, independently of us, Schubert postulated the same hypothesis [13], making use of a series of physicochemical equations.

in the body and, in particular, on the excretion of the metal from the body. The reason for this interdependence is competition between these metals and calcium ions, the concentration of which in the blood is very high, for the complexon. Under these circumstances the higher the value of K_{sup} of calcium by the metal (i. e. the greater its relative affinity for the complexon), the greater will be the degree of formation of the complex compound in the body and the more difficult it will be to break down.

Calculation of the values of 1g K_{sup} of calcium by yttrium, cerium and lead from its complexes with EDTA and DTPA gave the following results (the constants of stability of complexes of these metals with DDTE are not known):

	Y	Ce	Pb	Calculated by:
$CaNa_2EDTA$	7.0–7.3	5.3	7.3–7.6	[4; 14]
$CaNa_3DTPA$	9.5	9.5	8.0	[15; 16]

It will be clear from these results that, by comparison with EDTA, DTPA possesses a greater relative affinity for the metals tested; this may as a first approximation account for the more marked influence of DTPA on the magnitude of their deposition in the tissues and their excretion from the body, as is assumed, in particular, by Catsch [18]. In its efficacy, in the experiments with Y and Ce, DDTE occupies an intermediate position between DTPA and EDTA, on which basis it may be postulated that its K_{sup} is rather lower than that of DTPA.*

We examine this more closely, however, we discover a marked discrepancy: although there is a considerable difference in the efficacy of EDTA and DTPA in relation to lead (Table 3), the difference in their K_{sup} is very small. To explain this discrepancy it is evidently necessary to call in certain other parameters by which the two complexons can be distinguished. We know, for example, that the complexes of DTPA with metals are stable throughout a wider range of pH than are the EDTA complexes [17]; this may give DTPA an essential advantage in competition with the process of formation of lead hydroxide at the pH of the blood (lead peroxide begins to come down at about pH 6.0), which is a matter of great importance when the complexons are administered early. We also know [15] that DTPA forms double complexes of the M_2DTPA type; it is also possible that the velocity of the reaction of complex formation may also differ, and so on.

Of particular interest is the ability of the new complexons to prevent the deposition of cerium in bone tissue, in contrast to EDTA, which may even slightly increase its deposition in the skeleton. Catsch [18] explains this effect of DTPA by the much higher value of its K_{sup} for cerium, than that of EDTA; a similar explanation must evidently also apply to DDTE. Admittedly this is at present merely an experimentally established relationship, which does not reveal the direct cause of the decrease in the deposition of the metal in the bone tissue. In our earlier investigations [2] we tried to explain the slight increase in the deposition of cerium in the skeleton under the influence of EDTA by the fact that the complexon, besides combining the accessible molecularly dispersed colloid-like fraction and causing peptization of the finely dispersed fraction, delays the process of amalgamation of the colloidal particles of cerium, and thereby causes an increase of the concentration in the blood of particles of those dimensions which are deposited in the skeleton. If this is, in fact, true then it is possible that DTPA, which possesses a considerably higher affinity for cerium, competes successfully with the process of formation of the hydroxides of this metal in the blood, as a result of which it fixes a larger fraction of the Ce entering the body than does EDTA, and excretes it. This applies to a lesser degree to DDTE. In any case the problem of the connection between the K_{sup} and the ability to prevent the deposition of metals inthe bone tissue is one of great theoretical and practical interest. Wider investigations in this direction will demarcate more accurately the line of change from complexons a priori ineffective in lowering the deposition of cerium in the skeleton to those which are a priori effective.

* If this is, in general, acceptable, then on the basis of the results cited in this paper it can be postulated by interpolation that the constants of stability of the complexes of DDTE with Y and C are roughly 10^{19} and 10^{18} respectively.

In general it should be stressed that the physicochemical state of the various metals in the blood and the tissue fluid influences the efficacy of the complexons so strongly (which is clear from the figures cited here) that we can speak only of the close association [5] and not of proportionality between efficacy and K_{sup}. Attempts have recently been made to deduce equations which would take into consideration the principal factors influencing the efficacy of the complexons [13; 18] and which would satisfy the experimental findings. We may hope that the results described here will be useful in this direction.

Turning now to the discussion of the results of the late administration of the complexons, we must dwell on one very important fact. In previous work [2] we have demonstrated the good correlation existing between K_{sup} and the magnitude of the excretion (as a percentage of the quantity administered) of various metals in the urine when EDTA is administered early. This correlation is not observed if, as an index of efficacy, we take the relative increase, i. e. the degree of the multiplicity of the increase in excretion by comparison with control animals. This can be understood, for the complexon itself excretes a definite quantity of metal which is combined with it, and it does not stimulate the normal processes of its excretion from the body.

If we approach the results obtained relating to the delayed administration of complexons from this point of view, we may observe a relationship, which appears to us to be of theoretical importance, between K_{sup} and the fraction (percentage of the quantity administered) of the various metals excreted under the influence of complexons. For instance, from a comparison of Tables 4 and 6 it will be seen that EDTA increases the excretion of Y and Pb in the urine to roughly the same value (1.67 and 1.92 on the eighth day; 1.08 and 1;06 on the 13th day, and so on), irrespective of the fact that the level of excretion of Pb at the moment of injection of the complexon is 2-3.5 times higher than that of Y. Since the values of K_{sup} for Y and Pb are roughly equal for EDTA, it can be assumed that the value of K_{sup} does not determine the rate of increase but the absolute value of the excretion of the metal in the urine. This is well confirmed by the figures in the same tables showing the efficacy of DTPA (here only the 7th-8th and 37th-38th days should be compared, for at the other two periods of complexon injections the level of Y in the urine had not yet been normalized), where the excretion of yttrium (K_{sup} = = 9.5) reached a higher absolute value than the excretion of lead (K_{sup} = 8.0), in spite of the higher normal level of excretion of the latter.

It must be emphasized that, by using the absolute magnitude of the metal excreted by the complexon as indicator, we were on a sound physico-chemical footing which enabled us to reach the following two important conclusions. Firstly, the magnitude of excretion must depend on the dose, and on the stability of the complexon and of the complex compound formed in the conditions found in the body, and also on the accessibility to the complexon (whether physicochemical or histological) of the metal present in the tissues, blood and tissue fluid. Secondly, the higher the value of the K_{sup} possessed by the complexon, then other things being equal, the greater the fraction of the metal that it will excrete from the body. We can thus see that the general rule established in the experiments on early administration of complexons also applies when their administration is delayed. An important practical consequence of this is that synthetic chemistry places in our hands a definite physicochemical index which, in the first place, can guide our research, and in the second place, can determine the prospective therapeutic value of the new synthetic complexons.

Of the results of our present investigations into the delayed administration of complexons, we must mention above all the previously established [11] high efficacy of DTPA in the excretion of Ce. It is apparent from Table 5 that the roughly equal degree of increase in its excretion in the urine and feces is due principally to the mobilization of the isotope from the liver. Hence it may be concluded that the significantly higher efficacy of DTPA in the excretion of Ce than of the other metals is due to the accessibility of the fraction of the metal deposited in the liver. If this is so, then it is to be expected that DTPA will be much more effective in the excretion of "preferentially hepatic" [6] metals. To a lesser degree the same arguments apply also to DDTE. Such a sharp rise in the excretion of Ce in the feces is somewhat unexpected; this is due principally (so far as can be judged from the data in Table 5) to the direct transfer of Ce from the liver to the intestine.

A specific feature of both new complexons is the increased excretion of Y and Ce in the urine lasting 3-5 days or longer after their injection; in the experiments with lead, however, this specific feature was not observed. Meanwhile increased excretion in the feces takes place for Ce only under the influence of DTPA (lasting 3-4 days), but in case of Y – under the influence of both complexons (although only for the first day after their injection);

if the excretion of Pb in the feces is increased, then it is so only insignificantly and in isolated cases. We must point especially to the fact that an appreciable increase in the excretion of Y and Ce in the feces is observed usually after the first and sometimes after the second injection of complexon, i. e. at a time when the content of the metal in the liver is still quite high. It is still difficult to explain this prolonged action of the new complexons, although there are indications [19] of a slower excretion of DTPA than of EDTA from the body.

When comparing the efficacy of the complexons under trial, when administered early or late, attention must be directed to the following similarities and differences. A certain analogy is observed in the action of all the complexons tested, namely that the complexons which are more effective when administered early are also more active when given late. In the latter case, however, the differences in efficacy taking place with early administration are largely obliterated, and DDTE, which has a relatively weak action on the deposition of lead in the tissues, had practically no effect on its excretion from the body when given late. This smoothing out of the differences may be explained by the fact that in the late stages of the experiment only the readily soluble and biocomplex compounds of the metals [2] remain in the blood and tissue fluid, whereas at early periods after their entry into the body there is a complete assortment of different physicochemical fractions including hydroxides of different degrees of dispersion, with regard to which sharper variations appear both in the constants of stability and in the velocity of the process of peptization of the colloids of the different complexons.

Another fact to which attention must be drawn is the ability of the new complexons to increase the excretion of certain metals not only in the urine but also in the feces, which is of essential practical importance, for it relieves the load on the principal route of excretion, i. e. the kidneys, and makes them less likely to suffer injury by toxic metals and radioactive isotopes.

In conclusion there is need for special emphasis on an important fact in the treatment of poisoning by metals and radioactive isotopes. In experiments on rats DTPA gave the best results although, especially in the experiments with Pb, it was only slightly better than EDTA in its results. However, on the basis of the very interesting results of Laszlo and co-workers [20], according to whom complexes of metals with EDTA and DDTE are excreted in human urine in only half the quantity excreted in the urine of mice, whereas similar complexes with DTPA are excreted to the extent of 97.5% of that in mice, then it is to be expected that in man DTPA will be much more effective in clinical use than EDTA which is widely administered at the present time.

SUMMARY

The diaminodiethylester of tetraacetic acid(DDTE), diethylenetriaminepentaacetic acid-(DTPA) and ethylenediaminetetraacetic acid (EDTA) were tested for accelerating the elimination of yttrium, cerium and lead from the body.

With regard to yttrium and cerium the complexons can be arranged in the following series of decreasing efficacy both with early and delayed use: DTPA > DDTE > EDTA, and with regard to lead: DTPA > EDTA > DDTE. Following DDTE and DTPA injection increased elimination with urine and feces of yttrium and cerium, but not of lead, lasts several days. DTPA should be particularly efficient with regard to metals which are mostly deposited in the liver.

With delayed use of the complexons the calcium displacing constants ($K_{d[Ca]}$) correlate well with the absolute amount of the metal eliminated in the urine but not with the relative increase in its elimination. It seems justified therefore to extend the hypothesis of the relationship between ($K_{d[Ca]}$) and complexon efficacy from early to delayed use of these compounds.

A theoretical interpretation is presented of the differences in the efficacy of the above complexons.

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THE STUDY OF THE ACID-SOLUBLE PHOSPHORUS COMPOUNDS OF Penicillium chrysogenum Q-176 WHEN CULTIVATED IN DIFFERENT CONDITIONS

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In our previous work [1] we studied the metabolism of polyphosphates and of other phosphorus compounds during the submerged cultivation of Penicillium chrysogenum Q-176. We showed that the phosphorus metabolism in this organism is largely dependent on the conditions of cultivation and, in particular, on the composition of the medium. We showed, for example, that when corn extract was added to the usual synthetic medium, together with intensification of all synthetic processes there was a perceptible redistribution of the phosphorus among the fractions. We know that the acid-soluble, organic compounds of phosphorus such as, for example, free nucleotides and phosphorylated sugars, are of crucial importance to the whole phosphorus metabolism of living organisms. With regard to molds, however, very little work has yet been done [2-5] on the study of these highly important cell metabolites. Nevertheless it seemed likely to us that a more careful study of these particular phosphorus compounds might help to solve the problem of the influence of the composition of the nutrient medium on the phosphorus metabolism of the molds.

In the present investigation an attempt was made to study not only the composition but also the quantitative content of certain acid-soluble phosphorus compounds, and above all of the free nucleotides in P. chrysogenum, when cultivated on a medium with or without the addition of corn extract.

EXPERIMENTAL METHOD

Penicillium chrysogenum Q-176 was cultivated by submerged growth in Stone and Farrell's synthetic medium containing lactose, as described previously [1]. In a series of cases corn extract was added to this medium in a dose of 20 g dry weight per liter. Analysis showed that 1 g of this corn extract contained 23.34 mg phosphorus in the form of orthophosphate and only 4.76 mg in the form of stable organic compounds.

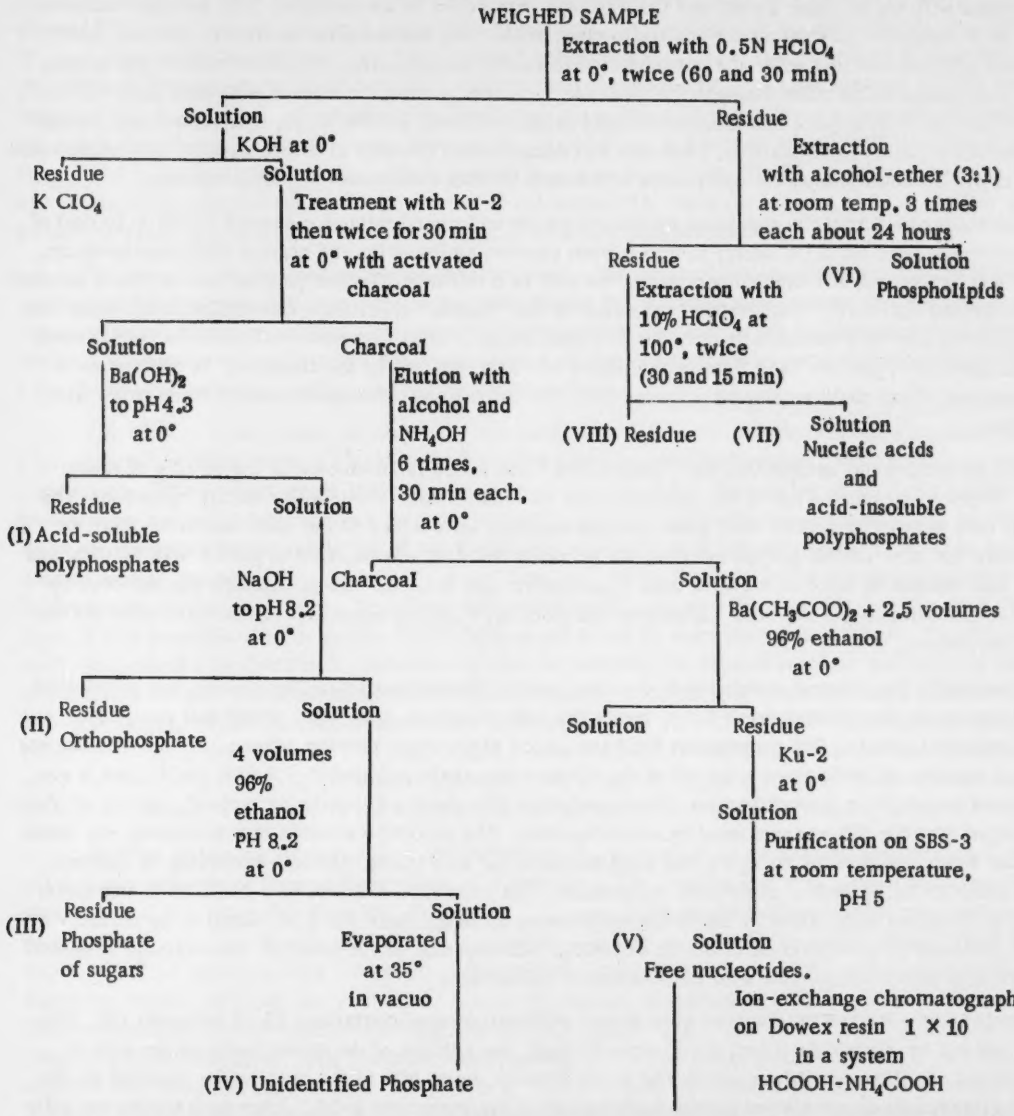
The mycelium of a 24-hour growth was subjected to chemical fractionation. With regard to the composition of the free nucleotides, we also analyzed the spores of the mold, and a 12-hour mycelium was used for collection of these. The collected material was carefully washed free from the elements of the medium with cold water, and dried lyophically.

Since in the performance of this investigation particular attention was directed towards the acid-soluble phosphorus compounds, we considered it necessary to conduct a series of trial experiments in order to select the most suitable and convenient method of extraction of this phorphorus fraction.

In the study of the acid-soluble phosphorus compounds, several extracting agents were employed. Most workers use for this purpose 0.25-1 N $HClO_4$ [5-7], although in other researches 5% TCA [8], 50-60% ethyl alcohol [9,10] and boiling distilled water [3] have been used successfully. We at once rejected the method of extraction of the material with boiling water, because of the possibility of thereby causing partial hydrolysis of the

labile compounds. During the comparison of the other three methods which we conducted with the mycelium of a 24-hour growth, it was found that extraction with alcohol extracted less than half the phosphorus compounds that could be extracted with the acids. This was thus considered unsuitable. During extraction of the mycelium with 5% TCA and 0.5N $HClO_4$ fairly similar results were obtained, although 7% more phosphorus passed into the TCA than into the chloric acid. In our work we nevertheless decided to use not TCA but 0.5N $HClO_4$, because it may subsequently be easily and quantitatively removed in the form of the potassium salt by neutralization with KOH solution in the cold [6]. Meanwhile the removal of TCA from the solution, usually carried out by means of sulphuric ether, is a very long and tedious task. After these and other trial experiments we decided on the following scheme of fractionation of the phosphorus compounds of P. chrysogenum.

Scheme of Fractionation of the Phosphorus Compounds of P. Chrysogenum Q-176


WEIGHED SAMPLE
Extraction with 0.5N HClO4 at 0°, twice (60 and 30 min)
Solution
KOH at 0°
Residue
Residue
K ClO4
Solution
Treatment with Ku-2 then twice for 30 min at 0° with activated charcoal
Residue
Extraction with alcohol-ether (3:1) at room temp. 3 times each about 24 hours
(VI) Solution Phospholipids
Residue
Extraction with 10% HClO4 at 100°, twice (30 and 15 min)
(VIII) Residue
(VII) Solution Nucleic acids and acid-insoluble polyphosphates
Solution
Ba(OH)2 to pH 4.3 at 0°
Charcoal
Elution with alcohol and NH4OH 6 times, 30 min each, at 0°
Residue
(I) Acid-soluble polyphosphates
Solution
NaOH to pH 8.2 at 0°
Charcoal
Solution
Ba(CH3COO)2 + 2.5 volumes 96% ethanol at 0°
(II) Residue Orthophosphate
Solution
4 volumes 96% ethanol PH 8.2 at 0°
Solution
Residue
Ku-2 at 0°
Solution
Purification on SBS-3 at room temperature, pH 5
(III) Residue Phosphate of sugars
Solution
Evaporated at 35° in vacuo
(V) Solution
Free nucleotides.
Ion-exchange chromatography on Dowex resin 1 × 10 in a system HCOOH-NH4COOH
(IV) Unidentified Phosphate

This scheme largely corresponds to that suggested by Zaitseva, Belozerskii et al. [8] for fractionation of the phosphorus compounds of Azotobacter vinelandii. The differences were that the acid-soluble phosphorus compounds were extracted by chloric acid instead of by TCA, the acid-soluble compounds were fractionated into fewer components, and at certain stages additional purification of the fractions with ion-exchange resins

was carried out (Ku-2 and SBS-3).

The lyophilically dried material (15 g) was thus extracted twice with 0.5N $HClO_4$ in the cold: the first time 450 ml of acid was used and the extraction lasted one hour, and the second time the volume of acid and the extraction time were both halved. The residue was washed three times with water and the washings were added to the extracts. After removal of the acid-soluble phosphorus compounds, the residue was treated three times at room temperature, each for 24 hours, with 300 ml of an alcohol-ether mixture (3:1) to remove the phospholipids fraction. The alcohol ether fraction was separated by centrifugation and the residue was twice extracted with 10% $HClO_4$ at 100°. For the extraction the volumes of acid taken were 450 and 225 ml respectively, and the duration of the first extraction was 30 minutes, the second 15 minutes. The residue was then carefully washed with water (three times) and the washings were added to the extracts. This fraction contained mainly the acid-insoluble polyphosphates and the nucleic acids. The residue after treatment with hot chloric acid was dried with alcohol and ether and not subjected to further fractionation. The acid-soluble phosphorus compounds were subjected to more thorough fractionation. For this purpose the extract with cold 0.5N $HClO_4$ was neutralized with KOH to pH 6 and allowed to stand in the cold until all the $KClO_4$ thus formed was precipitated. As shown by further investigations, when this was done a small quantity of orthophosphate was precipitated as well as certain unknown phosphorus compounds which were neither nucleotides or polyphosphates.

The neutralized solution was separated by centrifugation and passed through a column (1 cm × 10 cm) of the cation exchange resin Ku-2 (H^+ form) to free it from cations, amino acids and certain other contaminants. Purification was carried out at room temperature at the rate of 5 ml/min. To this purified and acidified solution was added activated charcoal* "karboraffin" (charcoal of the "Norite" type, from Czechoslovakia), which adsorbs quantitatively and very specifically only the free nucleotides. Trial experiments showed that under conditions a small quantity of pentoso- and fructosophosphates was also retained by the charcoal. In contrast to nucleotides, however, these could readily be removed from the charcoal by subsequent washing three times with distilled water.

In order to remove the nucleotides, the "karboraffin" was added in an amount of 1 g to 10 g of crude mycelium. Sorption was carried out in the cold twice for 30 minutes each time, with constant agitation. The charcoal was then thoroughly washed with water and the washings were added to the main solution. Next, in order to remove the acid-soluble polyphosphates, the pH of the solution was adjusted to pH 4.5 with $Ba(OH)_2$ and the solution was allowed to stand in the cold until a precipitate had formed. The precipitate was removed by centrifugation, and after its pH had been adjusted to 8.2 with NaOH, it was again allowed to stand until all the precipitate had formed.

The precipitate thus formed, consisting in our case mainly of orthophosphate (Residue II), was redissolved in 0.1N HCl and again precipitated at pH 8.2 to purify the orthophosphate from other phosphorus compounds which were deposited with it. The supernatant fluid was added to the main solution, after which the mixture was treated with 4 volumes of 96% ethanol, the pH of the solution was again adjusted to 8.2 with NaOH, and it was allowed to stand overnight in the refrigerator. The precipitate thus obtained, consisting probably mainly of phosphorylated sugars (Residue III) was separated by centrifugation. The alcoholic solution thus remaining was freed from Ba^{++} ions by the addition of 1N H_2SO_4 and evaporation at 35° to a small volume. According to Umbreit, this fraction contains little-studied phosphorus compounds. The Ba residue obtained as a result of fractionation was returned to a soluble state either by treatment with cation exchange resin Ku-2 (H^+ form) or by redissolving in 0.1N HCl, followed by precipitation of the Ba as $BaSO_4$. The reaction of the solutions was adjusted to neutral and they were kept in the refrigerator with the addition of chloroform.

The nucleotides adsorbed on charcoal were eluted with 50% ethanol containing 1% of ammonia [9]. Elution was carried out by mixing six times, for 30 minutes each, the volume of the eluent being at the rate of 10 ml/g charcoal. In these conditions, according to our findings, up to 80% of the nucleotides adsorbed on the charcoal was eluted; subsequent elution gave a further yield of not more than 1-2%. After each elution the solution of nucleotides was filtered free from charcoal through a No. 4 glass filter with a 1 cm layer of asbestos. The addition of asbestos led to the complete freeing of the solution from the presence of fine charcoal dust.

* Before use the charcoal was boiled for one hour in 2N HCl, washed with water until the reaction was neutral, and dried in a cupboard at 105°.

To the solution thus purified, after neutralization with HCl, was added 2.5 volumes of alcohol and Ba acetate until complete precipitation of the barium salts of the nucleotides from the solution [8]. Usually, in order to secure complete precipitation of nucleotides, the solution was left overnight in the refrigerator. The residue thus obtained was redissolved by shaking its aqueous suspension with cation exchange resin Ku-2 (H^+ form). Pigments, which were present in small quantity in the solution, were removed by means of action exchange resin SBS-3 (NH_4^+ form). For this purpose the solution of nucleotides was passed through a column (1 cm × 5 cm) of the above resin at a velocity of 2ml/min.

After removal of the pigments, the pH of the solution was adjusted to pH 7-9, and ion-exchange chromatography was subsequently carried out. In all the fractions obtained (from I to VIII, see Scheme), the various forms of phosphorus were determined, the method of Berenblum and Chain, as modified by Weil-Malherbe and Green [12], being used for determination of the phosphorus. In fractions I-V we determined: the orthophosphate (P_{ortho}) directly in the solution; the "10-minute" phosphorus (P_{10}), i. e. the phosphorus hydrolyzed to orthophosphate in 10 minutes in 1N HCl at 100°, and the total phosphorus (P_{total}) after ignition in 0.25 ml concentrated $HClO_4$ at 150-180°. The labile phosphorus (P_{lab}) was found by the difference between P_{10} and P_{ortho}, and the stable phosphorus by the difference between P_{total} and P_{10}. In fractions VI and VIII we determined only P_{total}, and in the hot chloric extract (VII), besides P_{total} we determined the P_{ortho} formed principally on account of acid-insoluble polyphosphates in the process of extraction [1]. Thus the amount of P_{ortho} in this fraction could be used to judge approximately the amount of acid-insoluble polyphosphates.

Fractions III, IV, V were studied in greater detail. For instance, an attempt was made to identify the organic phosphorus compounds contained in fractions III and IV. For this purpose these fractions were studied by means of paper chromatography. Chromatographic separation was carried out on Leningrad slow paper, saturated with a 1 % solution of boric acid in 60% ethanol, in a solvent of: methanol-concentrated ammonia-2% boric acid (12 : 2 : 3) [13] in both ascending and descending directions.

The chromatograms were developed by the methods of Hanes and Isherwood [14], Partridge [15] and Rorem [16]. Rorem's method, using a 1% solution of quinine sulfate in absolute ethanol, was found to be particularly convenient. This reagent reacts with phosphates to give white stains, fluorescing in UV light against a grey background, and is readily removed from the paper by treatment with absolute ethyl alcohol. In some cases, when the quantitative determination of the phosphates separated on the paper was required, after removal of the quinine sulfate the corresponding stains were cut from the chromatograms and eluted overnight with 0.1N HCl. In the eluates we determined the phosphorus [12], ribose [15], glucose [15], and, if this compound absorbed UV light, for its identification its spectra in UV light at pH 1 and 11 were also photographed. The most detailed study was reserved for fraction V, consisting of free nucleotides. For separation of the individual components of this fraction we used ion-exchange chromatography in columns containing anion exchange resin Dowex-1 (×10; 200-400 mesh). Before use, the Dowex-1 was first washed with 2N HCOOH in 2N NaCOOH (to completely remove Cl^-), then with 85% HCOOH and, finally, with bidistilled water until the reaction to litmus was neutral. At the end of the work, in order to regenerate the resin, the upper dark layer (about 2 mm) was discarded and the remainder of the resin in the column was washed with 85% HCOOH and water.

Sorption of the nucleotides contained in fraction V was carried out in a column measuring 1 × 18 cm at pH 7-9 at a velocity of flow of the solution of 1 ml/min. The column was then washed with distilled water until the washings ceased to show UV absorption. To separate the nucleotides sorbed on the column, the method of gradient elution in a formic acid system devised by Hurbert et al. [6,7] was used. Into a mixer was placed 250 ml distilled water, and for collection of fractions 4 ml in volume we used the mechanical collector of a Struchkov system [18], and the velocity of flow of the solvent through the column was 0.5 ml/min. In each fraction we measured the absorption in UV light at 260 mμ against water, and on the basis of the results obtained we constructed elution curves. The fractions corresponding to the individual peaks on the elution curves were pooled together and dried in vacuo at room temperature. From certain fractions, especially those containing a large quantity of ammonium formate, the nucleotides were extracted with the aid of "karboraffin" activated charcoal with subsequent elution of the nucleotides with ammoniacal ethanol, as described above. The nucleotides were identified by: their position on the elution curve [6]; the determination of the various forms of phosphorus; the determination of ribose [15]; the determination of hexose with anthrone [15]; the behavior during electrophoretic separation on paper in acetate and borate buffers, parallel with known compounds [8]; the value of R_f during chromatographic separation [19,20] before and after hydrolysis in 1N HCl at 100° for one hour; the absorption

spectrum in UV light in 0.1N HCl (pH 1) and in 0.1N KOH (pH 11) of the eluates from the electrophoregrams and chromatograms (elution of nucleotides and nitrogenous bases from the paper was carried out with 0.1N HCl overnight). To identify the nucleotides from the spectra the following ratios were calculated: $\frac{E_{250}}{E_{260}}$; $\frac{E_{270}}{E_{260}}$; $\frac{E_{275}}{E_{260}}$; $\frac{E_{280}}{E_{260}}$; the values thus obtained were compared with known values from the literature [6, 21, 22]. The individual nucleotides were calculated quantitatively in micromoles, using the corresponding coefficients for this purpose [21,22].

EXPERIMENTAL RESULTS AND DISCUSSION

The results showing the distribution of the various forms of phosphorus in all the fractions which we obtained from a 24-hour mycelium of P. chrysogenum Q-176, when cultivated in a medium with or without corn extract are given in Tables 1 and 2. The analytical figures given in Table 1 are calculated per g dry weight of mycelium. However, in view of the fact that the growth of the mass of mycelium in a medium with corn extract was much (4.4 times) greater than on a medium without it, it appeared interesting to calculate the content of phosphorus compounds also per 100 ml of medium (Table 2). On examination of Table 1, it will be noted firstly that the P_{total} content in the mycelium cultivated in a medium with corn extract, when calculated per g dry weight weight, was smaller than when the mold was cultivated in a pure synthetic medium. The qualitative composition of the phosphorus compounds of all the fractions which we obtained in both sections of the investigation, however, was absolutely identical, and thus it had little to do with the composition of the nutrient medium. Meanwhile the addition of corn extract to the medium had a strong stimulating effect on the growth and division of the cells, which led to a considerable intensification of the process of absorption of phosphorus from the medium and of its utilization in various synthetic processes.

It can be seen from Table 2 that the mycelium cultivated in a medium with corn extract contained far more of all the phosphorus compounds, when calculated per 100 ml of medium, than in the other variant when the mycelium was cultivated in ordinary synthetic medium. It is interesting to note that in spite of this fact, the content of all the phosphorus fractions in the two cases, when calculated per g dry weight (Table 1), nevertheless remained more or less at the same level. It may be observed from the figures in Table 2 that acid-soluble polyphosphates were completely absent in both variants, which may be judged by the absence of appreciable quantities of P_{lab} in the fraction precipitated by Ba salts at pH 4.3. This fact is in complete agreement with our previous conclusion [1] regarding the absence of polyphosphatases of depolymerizing type in molds during submerged cultivation, capable of splitting higher polymer, acid-insoluble polyphosphates into low-polymer, acid-soluble polyphosphates. So far as the acid-insoluble polyphosphates are concerned, judging by the quantity of P_{ortho} in the hot $HClO_4$ extract, they were present at this time in the mycelium in considerable amount. Moreover, in complete agreement with our previous results [1], in the case of cultivation of P. chrysogenum in a medium with corn extract, the mycelium contained much less of the acid-insoluble polyphosphates than in the variant obtained in the ordinary medium. The same may also be said of the relative proportions of P_{ortho}. It must be pointed out that when the total quantity of P_{ortho} in the mycelium was calculated, apart from the fraction precipitated with Ba^{++} at pH 8.2 and consisting in our material solely of P_{ortho}, we had to take into consideration also the phosphorus precipitated along with the $KClO_4$ during the neutralization of the cold chloric extract. According to our preliminary findings, this consisted largely of orthophosphate.

The fact that a relatively smaller content of P_{ortho} and acid-insoluble polyphosphates was present in the mycelium cultivated in a medium with corn extract was evidently associated with the far more energetic utilization of these compounds in the various synthetic processes which, as has been shown, take place very actively in these conditions. It must also be pointed out that the addition of corn extract to the medium (creating better conditions for the whole metabolic activity of the mold) has the greatest stimulating effect on the formation of phospholipids; the content of the latter in the corresponding variant was almost twice as high as in the control (Table 1). So far as the other phosphorus compounds are concerned, and especially such very important metabolites as the phosphorylated sugars, the free nucleotides and the nucleic acids, their content in the mycelium, in spite of the quite different level of growth of its mass in the two cases, remained suprisingly constant. This fact confirms once again the existence of a close connection between the metabolism of these compounds and the intensity of the growth and division of cells.

TABLE 1. Content of Different Forms of Phosphorus in the Mycelium of P. chrysogenum Q-176 when Cultivated in a Medium with and without Addition of Corn Extract. (Results in μg P/g dry weight)

Fraction	Pure synthetic medium				Synthetic medium with corn extract			
	P_{ortho}	P_{lab}	P_{stab}	P_{total}	P_{ortho}	P_{lab}	P_{stab}	P_{total}
Extract with 0.5N $HClO_4$ at 0-4°								
Residue obtained after neutralization of extract with KOH	—	—	—	3398.0	—	—	—	2910.0
Free nucleotides	—	8.5	56.7	65.2	—	8.3	52.5	60.8
Residue obtained after precipitation with Ba^{++}, pH 4.3	148.0	0.0	0.0	148.0	74.0	8.0	67.0	149.0
The same, pH 8.2	3680.0	0.0	0.0	3680.0	1920	0.0	0.0	1920.0
The same after addition of alcohol	116.0	22.0	300.0	438.0	118.0	12.0	281.0	411.0
Fraction not precipitated with Ba^{++} at pH 8.2 with addition of alcohol	0.0	0.0	346.0	346.0	0.0	0.0	515.0	515.0
Alcohol-ether extract	—	—	—	635.0	—	—	—	1060.0
Extract with 10% $HClO_4$ at 100°, 30 min	6450.0	—	9750.0	14 200.0	3200.0	—	9300.0	12 500.0
Residue after all extractions	—	—	190.0	190.0	—	—	174.0	174.0
Original mycelium	—	—	—	23 100.0	—	—	—	19 700.0

TABLE 2. Content of Different Forms of Phosphorus in the Mycelium of P. chrysogenum Q-176 when Cultivated in a Medium with and without Addition of Corn Extract. (Results in μg P/100 ml of medium)

Fraction	Pure synthetic medium				Synthetic medium with corn extract			
	P_{ortho}	P_{lab}	P_{stab}	P_{total}	P_{ortho}	P_{lab}	P_{stab}	P_{total}
Extract with 0.5N $HClO_4$ at 0-4°								
Residue obtained after neutralization of extract with KOH	—	—	—	339.8	—	—	—	1280.0
Free nucleotides	—	0.8	5.7	6.5	—	3.7	23.1	26.8
Residue obtained after precipitation with Ba^{++}, pH 4.3	14.8	0.0	0.0	14.8	32.6	3.5	29.5	65.6
The same, pH 8.2	368.0	0.0	0.0	368.0	844.8	0.0	0.0	844.8
The same after addition of alcohol	11.6	2.2	30.0	43.8	51.9	5.3	123.6	180.8
Fraction not precipitated with Ba^{++} at pH 8.2 with addition of alcohol	0.0	0.0	34.6	34.6	—	—	226.6	226.6
Alcohol-ether extract	—	—	—	63.5	—	—	—	466.4
Extract with 10% $HClO_4$ at 100°, 30 min	645.0	—	975.0	1420.0	1408.0	—	4092.0	5500.0
Residue after all extractions	—	—	19.0	19.0	—	—	76.6	76.6
Original mycelium	—	—	—	2310.0	—	—	—	8668.0

Whether growth of the culture took place in a medium with or without the addition of corn extract, the mycelium contained a negligible amount of free nucleotides. In 1 g of the material there was not more than 0.6-0.65 mg of these compounds (calculated as phosphorus). It is interesting to note that, by comparison with

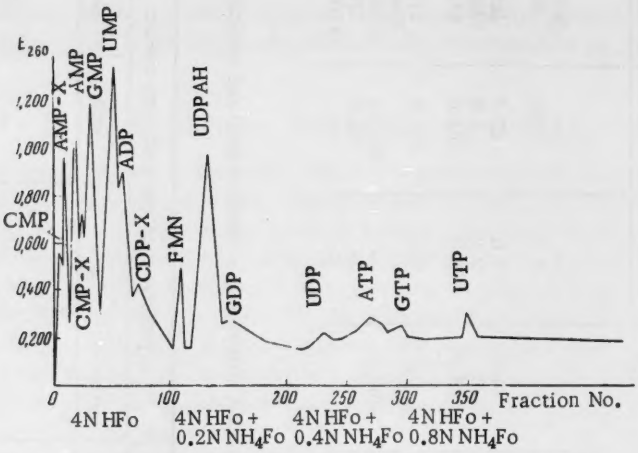


Figure. Separation of acid-soluble nucleotides of a 24-hour mycelium of P. chrysogenum Q-176, cultivated in synthetic medium without addition of corn extract. Separation by means of ion-exchanging chromatography on Dowex 1×10(200-400 mesh), in a formate system. (Fo = COOH.)

TABLE 3. Content of Free Nucleotides in the Mycelium of P. chrysogenum Q-176 with Different Conditions of Cultivation. (I: in μmole/15 g dry weight; II: in μmole/15 liters of medium)

Nucleotides	Pure synthetic medium		Synthetic medium with corn extract		Nucleotides	Pure synthetic medium		Synthetic medium with corn extract	
	I	II	I	II		I	II	I	II
CMP	Present in very small amount				UDPAH	2.50	2.50	1.55	6.82
AMP-X	0.53	0.53	0.80	3.52	GDP	Not detected			
AMP	2.40	2.41	2.33	10.25	UDP	0.06	0.06	0.61	2.68
CMP-X	0.20	0.20	0.13	0.57	ATP	0.64	0.64	0.97	4.27
GMP	1.57	1.57	0.61	2.68	GTP		Present in traces only		
UMP	3.50	3.50	2.15	9.46	UTP		Present in traces only		
ADP	0.90	0.90	0.99	4.36	UDP-hexose-I	26.00	26.00	6.20	27.30
CDP-X	Not detected		0.33	1.45	UDP-hexose-II	18.00	18.00	5.00	22.00
FMN	Not detected				UDP-oligo-saccharide	Not detected		18.10	80.00

bacteria [8], the content of free nucleotides in the molds was many times smaller. Moreover, on account of the extremely low value of P_{lab} of this fraction, it may be supposed that in its composition there was a very small quantity of nucleoside di- and tri-phosphates, which are known to play a crucial role in the whole metabolism of the cell [17]. This conclusion was fully confirmed by the more detailed study of this fraction by means of ion-exchanging chromatography (figure , Table 3).

The figure shows the elution curve of the free nucleotides obtained from the mycelium cultivated in the ordinary synthetic medium. Each peak shown on the elution curve was carefully identified as shown above. Many such elution curves were obtained for nucleotides cultivated in a medium with corn extract also. However, since the curves were almost identical in both cases, we do not reproduce them here. Examination of the figure

shows that the mycelium of P. chrysogenum Q-176 contained a fairly wide assortment of nucleotides, which remained qualitatively unchanged when the mold was cultivated in the different nutrient media (Table 3). In both variants investigated we found: nucleoside-5-monophosphates–cytindinemonophosphate (CMP), AMP,GMP, and UMP; nucleoside-5-diphosphates – ADP, GDP, and UDP; and finally, nucleoside-5-triphosphates – ATP, GTP, and UTP. The following were also identified: FMN and uridinediphosphate acetylhexosamine (UDPAH), and also certain unknown derivatives of AMP, CMP and cytidinediphosphate (CDP). It is possible that the derivatives of CMP and CDP were complexes with the peptides of the corresponding nucleotides, the presence of which in molds was recently established by Bergkvist [24].

The greater part of the nucleotides in the mycelium was present in the form of monophosphates. In the mold cultivated in pure synthetic medium, these accounted for about 65% of all the free nucleotides. Nucleoside di- and triphosphates were present in extremely small amounts. Analysis of the nucleotide composition of the condiospores of P. chrysogenum Q-176, which we made for the purpose of comparison with the mycelium, gave roughly the same qualitative picture. In the spores, however, in contrast to the mycelium, a relatively small quantity of nucleoside monophosphates and UDPAH was present. Returning to the mycelium, it must be pointed out that in the medium without corn extract roughly 50% of the nucleotides were uridyl derivatives, 30% adenyl and 15% guanyl, and only about 5% were cytidyl derivatives. Comparison in this respect of the mycelium grown in a medium with and without corn extract showed that the quantitative proportions of the derivatives of the various nucleosides in the two mycelia were somewhat different. The relative proportions of adenyl and uridyl derivatives, for example, were changed. In the mycelium obtained when corn extract was added to the medium, there was a far higher content of adenyl derivatives, and in quantity they were roughly equal to the content of uridyl derivatives. It was characteristic that the increase in the content of adenyl derivatives in this case took place mainly at the expense of the more highly phosphorylated nucleotides. After careful examination of the figures shown in Table 3 it can be seen that the mycelium grown in a medium with corn extract in general contained rather more nucleoside di- and triphosphates per g dry weight. This was evidently associated with the general activation of all the synthetic processes taking place under these conditions of growth.

It must also be pointed out that when the mold was cultivated in a medium with corn extract, a marked increase was observed in the synthesis of the nucleotides themselves, the content of which, calculated in terms of a definite volume of nutrient medium, became much increased for all the nucleotides. The velocity of synthesis of the majority of nucleotides in a medium with corn extract, however, stayed slightly less than the velocity of growth of the mass of mycelium, leading to lower values of their content when calculated per g dry weight. It is interesting that the only exception in this sense was the adenyl derivatives, especially ADP and ATP, the content of which per g dry weight in the mycelium grown in a medium with corn extract became even higher. This fact affords additional confirmation of the importance of the ADP-ATP system in the intensification of all synthetic processes.

All the results mentioned above referred to the nucleotides adsorbed from the acid-soluble fraction on to activated charcoal, followed by elution and chromatographic separation on ion-exchange columns. It was found, however, that not all the nucleotide derivatives were adsorbed in our experiments on the activated coal. Some part of the derivatives of uridylic acid remained in solution under these conditions of sorption, and we found them in other fractions. For instance, during the chromatographic study of the fraction precipitated from the cold $HClO_4$ extract with Ba on the addition of alcohol (fraction III), both variants investigated showed, besides a small amount of phosphates of sugars which we did not identify, two compounds giving absorption in UV light.

By paper chromatography in the solvent suggested by Sobolev et al. [13], these compounds were separated into two distinct stains, which after elution with 0.1N HCl and subsequent study, were found to be derivatives of uridylic acid, containing for 1 mole of uridine 1 mole of ribose, 2 moles of phosphorus and 1 mole of hexose, giving a positive reaction with anthrone and aniline phthalate. It may thus be considered that in this case we were dealing with derivatives of the UDP-hexose type, differing from each other by the composition of the sugar present. Besides these two substances, in both variants of the experiment we found one further compound of nucleotide nature, which was not precipitated by Ba and alcohol from the cold $HClO_4$ extract and which we discovered during the chromatographic study of the alcoholic solution containing, according to Umbreit, little-studied phosphorus compounds (fraction IV). After evaporation of the alcohol, the concentrated solution was applied to paper and subjected to chromatographic separation in the solvent of Sobolev et al. Analysis of the chromatograms thus obtained showed that this fraction in both variants of the experiment contained the same assortment of phosphorus compounds, which we did not identify. One of them gave absorption in UV light, and after elution with 0.1N HCl, it was studied in more detail. This compound also was a derivative of uridylic acid, containing for 1 mole of uridine 1 mole of ribose, 2 moles of phosphorus and more than 2 moles of hexose. This compound

thus differed from the first two, isolated from fraction III, only by its significantly greater content of hexose. It seems probable that the first two uridyle derivatives were UDP-glucose and UDP-galactose, which were detected in P. chrysogenum Q-176 by Ballio et al. [4]. So far as the third uridyl derivative is concerned, obtained from the alcoholic solution, it is not excluded that it could be the trisaccharide, discovered by Ballio and Russi [25] in a lactose-containing medium during cultivation of P. chrysogenum therein, activated by means of UDP. It is interesting to note that this trisaccharide, made up of one molecule of glucose and two molecules of galactose, was formed in large amounts by means of the reaction of transglucosidation of lactose, which is known to be an essential component of the medium for cultivation of P. chrysogenum. It may be supposed that the formation of this trisaccharide is necessary for the synthesis of the polysaccharides of P. chrysogenum, which are known to include in their composition glucose and galactose as well as glucosamine [26]. Thus the UDP-activated oligosaccharide which we found, like all the other uridyl derivatives of sugars found in P. chrysogenum, might be an intermediate product in the process of biosynthesis of the cell polysaccharides. During the quantitative estimation of these uridyl derivatives (Table 3) in both variants it was established that they were present in the mycelium in enormous quantities. This applied especially to the mycelium grown in the ordinary nutrient medium, where their content was many times greater than the total content of all the other nucleotides together. The content in the mycelium was highest of that very UDP-oligosaccharide which, unfortunately, was determined quantitatively only in the first case (Table 3).

It is interesting to note that when the mold was cultivated in a medium with corn extract, the content of these uridyl derivatives in the mycelium, calculated in terms of dry weight, was far less. In this variant of the experiment, as we have already pointed out, all the uridyl nucleotides were present in smaller quantities than in the mycleium grown in the ordinary synthetic medium. It was characteristic that when the content of UDP-hexose was calculated in terms of a definite volume of medium, it was found that the figures obtained in the two variants were roughly the same. In our opinion, this fact may indicate indirectly that the stimulating action of corn extract on the growth and development of P. chrysogenum has little effect on the process of biosynthesis of polysaccharides, which is known to involve the participation of uridyl coenzymes [9].

In conclusion it must be pointed out that in the present research we found no trace whatsoever in the mycelium, grown in a medium with corn extract, of the unknown stable phosphorus compounds which we previously found in large amounts in P. chrysogenum Q-176 when cultivated inder similar conditions [1]. This fact evidently depends on the presence of a very small amount of phytin in the batch of corn extract used in the present investigation, which was not present in the previous research. Furthermore, we know from the literature [2; 23] that the content of phytin in the medium has a strongly stimulating effect on the accumulation of phosphorus compounds in the mycelium of molds. In the analysis of the results showing the influence of corn extract on phosphorus metabolism in a mycelium developing in a medium containing it, it is very important to take into consideration the composition of this corn extract and, in particular, of the composition of the phosphorus compounds which it contains.

SUMMARY

Acid-soluble phosphorus compounds have been studied in P. chrysogenum Q-176 in submerged cultures in a pure synthetic medium and with added corn extract. Particular attention was paid to acid-soluble nucleotides. In a 24-hour mycelium of P. chrysogenum these are very insignificant. Not more than 0,65 mg of these major phosphorus compounds are contained in 1 g dry material. Among the components of this fraction the following have been identified: CMP, AMP, GMP, UMP, FMN, ADP, UDP, GDP, ATP, GTP, and UTP.

It was shown that most of the mononucleotides belong to nucleoside monophosphates. In addition to free mononucleotides a number of derivatives of AMP, CMP, CDP and UDP have been found in the mycelium of P. chrysogenum. Great amounts of UDP derivatives have been detected, viz., uridinediphosphate acetlyhexosamine, 2 UDP hexoses, and especially a hitherto unknown UDP oligosaccharide consisting of several different monoses. The content of UDP derivatives is so high that it exceeds séveralfold all the other acid-soluble nucleotides together.

Addition of corn extract to the synthetic medium results in marked increase of adenyl nucleotides synthesized in the mycelium, ADP and ATP in particular. This is presumably linked with the general increase in the rate of growth processes caused by the corn extract, and first of all with the concomitant increase in synthesis of nucleic acids. However, corn extract does not affect the accumulation of the UDP-derivatives of hexoses.

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THE ACTION OF CHLORAMPHENICOL ON THE PHOSPHORUS AND NUCLEAR METABOLISM OF Azotobacter vinelandii

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Chloramphenicol specifically inhibits protein synthesis, whereas its action on nucleic acids depends on the concentration of the antibiotic and on the species of bacteria [1-4]. Bernlohr and Webster [5] studied the influence of chloramphenicol on the incorporation of C^{14}-glycine into RNA, DNA and protein of Azotobacter agile, and found that high concentrations of the antibiotic (10^{-2}M and 10^{-3}M) inhibit the synthesis of all these compounds, whereas lower concentrations (10^{-4}M–10^{-6}M) inhibit the incorporation of C^{14}-glycine into protein and DNA only, the synthesis of RNA continuing.

A series of researches has shown that in the presence of chloramphenicol an unstable, metabolically inactive RNA is formed in the cells of certain bacteria [2,4,6-8], differing from the RNA of normal cells. In this connection it is extremely interesting to compare the nucleic acids in normal cultures and in those inhibited by the antibiotic.

The object of the present work was to investigate the influence of chloramphenicol on the various substances of the cell and, in particular, on the metabolism and composition of the nucleic acids of Azotobacter vinelandii. An attempt was also made to separate the ribonucleoporteins of normal and chloramphenicol-inhibited cultures of Azotobacter and to determine any possible differences in their properties.

For this work we used a strain of Az. Vinelandii (from the museum of the department of microbiology of Moscow State University), grown on a modified Burke's medium [9]. Special experiments showed that equal concentrations of chloramphenicol * had the strongest bacteriostatic action within limits on the younger Azotobacter cells. In a concentration of $5 \cdot 10^{-4}$M in the medium, chloramphenicol almost completely arrested growth of Az. vinelandii when in the initial logarithmic phase of development (15-hour culture). In such a culture inhibited by chloramphenicol cell division is disturbed, and consequently the cells increase in size by comparison with normal by roughly 50%. We chose the 6-hour period for the action of chloramphenicol, for during this time in normal conditions the number of cells in a 15-hour culture of Azotobacter on the average doubled.

After cultivation for 21 hours radioactive orthophosphate (0.5 mC $K_2HP^{32}O_4$/ liter of medium) was added to both normal Azotobacter cells and to those inhibited with chloramphenicol during the last 6 hours of growth, and after incubation for 5, 15 and 30 minutes samples were withdrawn for measurement of the radioactivity of the various forms of phosphorus. In the case of the action of chloramphenicol, the bacterial mass was washed free from radioactive orthophosphate with medium containing antibiotic. In addition, the respiration, oxidative phosphorylation [10] and incorporation of C^{14}-glycine (100,000 imp/ min/ ml medium) into the proteins of Az. vinelandii were measured in Warburg flasks for periods of 5, 15 and 30 minutes at 30° and a rate of 120 oscillations/ min. After incubation with labeled glycine, the cells were throughly washed with 5% TCA at first in the cold and then after warming to 90° to remove the free amino acids, mononucleotides and nucleic acids. The

* The authors of this paper express their gratitude to professor G.F.Gauze for his kindness in making available a highly purified preparation of chloramphenicol.

residue was adjusted to constant weight and its protein content determined. The relative radioactivity (RA) of C^{14}-protein was calculated in imp/min/mg protein or per 10^9 cells.

The method of fractionation of the phosphorus compounds and of measurement of their RA were as described in a previous communication [11]. After hydrolysis with acid (1N HCl, 7 and 30 minutes at 100°) or ignition, the phosphorus was determined by the method of Berenblum and Chain as modified by Weil-Malherbe and Green [12].

The total (P_{total}) and labile (P_{lab}) phosphorus contents of the monucleotides were estimated as the difference between the total and 7-minute values of the phosphorus before and after adsorption of the nucleotides on charcoal (Karboraffin, Czechoslovak manufacture). The soluble polyphosphates were determined after precipitation with Ba^{++} ions at pH 4.5, and the phosphoric esters of carbohydrates likewise at pH 8.2 with the addition of four volumes 96% alcohol. The acid-insoluble phosphorus compounds were separated into RNA and DNA fractions by means of a slight modification of the method of Schmidt and Tanhauser [13]. During the study of the nucleotide composition of RNA by means of paper electrophoresis [11], and of the DNA bases by means of paper chromatography, we employed the numerical coefficients cited in the literature [14]. The total RNA was determined by addition of the amounts of the four component nucleotides, and the radioactivity of the total RNA P by summation of the radioactivity of the P of its nucleotides. DNA was determined spectrophotometrically [15], and also by Dische's chemical method, as modified by Burton [16]. Measurement of the radioactivity of the P-DNA was carried out in preparations of its Na salts [11]. The protein was determined by the biuret reaction [17] and by the method of Lowry et al. [18], the nitrogen by the micro-Kjeldahl method, polysaccharide as glucose by the anthrone method [19], and the amino nitrogen of the free amino acids by the method of Yemm and Cocking [20].

The ribonucleoproteins from the extracts of the normal, restored and chloramphenicol-inhibited cultures of Azotobacter were separated by the method of electrophoresis on starch gel [21]. The restored culture was obtained by removal of the inhibiting action of the antibiotic by washing the inhibited cells for three hours with normal medium.

In order to measure the RA of the P-nucleoproteins of the normal and inhibited Azotobacter cells, the corresponding culture was incubated in a medium containing $K_2HP^{32}O_4$ (0.5 mC/liter of medium) for two hours. The Azotobacter cells were washed with water and destroyed by thorough grinding in a mortar with two parts by weight of powdered glass. The degree of destruction of the cells was controlled by microscopic examination of the specimens. Extracts were prepared in a phosphate buffer of the following composition: $1.6 \cdot 10^{-3}$M K_2HPO_4 and $0.4 \cdot 10^{-3}$M KH_2PO_4 in the presence of Mg^{++} ions in a concentration of $5 \cdot 10^{-3}$M $MgSO_4$ [22]. The glass and undestroyed Azotobacter cells were separated by centrifugation on MSE at 3000 g, followed by clearing of the extracts at 20,000 g. All operations were carried out strictly in the cold.

To separate the ribonucleoproteins (RNP), 2 ml of extract containing 1.0-1.5 mg of nucleic acids was applied to the starch gel on the column. The electrophoretic separation lasted 17 hours at 400 v, 60 ma, in a tris buffer at pH 7.4 and μ 0.020. Cooling was by means of tap water. After electrophoresis, the column was cut up into 60 parts of equal weight, which were frozen at -20°. After thawing, the filamentous gel was squeezed almost dry with a plunger in cylindrical, thick-walled tubes. The volume of solution in each segment of gel was 3 ml with small variations (± 0.2 ml), which were taken into consideration when calculating the amount of RNA. From each fraction 0.5 ml of solution was poured into a centrifuge tube, diluted ten times with 5% $HClO_4$ and hydrolyzed for 30 minutes at 100°, and after centrifugation, the optic density of the centrifugate was determined in the SF-4 spectrophotometer at 270 and 290 mμ. On the basis of the difference in the values of the optic density ($E\Delta_{270-290}$) of all the fractions, an absorption curve was constructed (figure). The limits of each nucleotide peak were marked in accordance with the minimal values of E.

The RNA content of each peak (in μg) was calculated by the formula:

$$\frac{\Sigma[(\Delta 270-290)\cdot V]\cdot a}{0.19}\cdot 10.43,$$

where (Δ270-290) is the difference in the optical densities at 270 and 290 mμ of each fraction of the particular peak; V the volume of each fraction of that peak; a the dilution of the fractions; 10.43 = 100/9.59, where 9.59 is the phosphorus content in % in the RNA of Az. vinelandii. Furthermore, in each of the three peaks obtained we

determined the protein [18], DNA [16] and RNA by the reaction with orcin [19]. In the first and second peaks, which were ribonucleoproteins (RNP_1 and RNP_2), we calculated the ratio RNA/protein. For measurement of RA P-RNP we selected the fractions with the highest optic density. After ignition of RNP with concentrated $HClO_4$, the orthophosphate and its relative activity (P-RNP) were determined by the usual method [11].

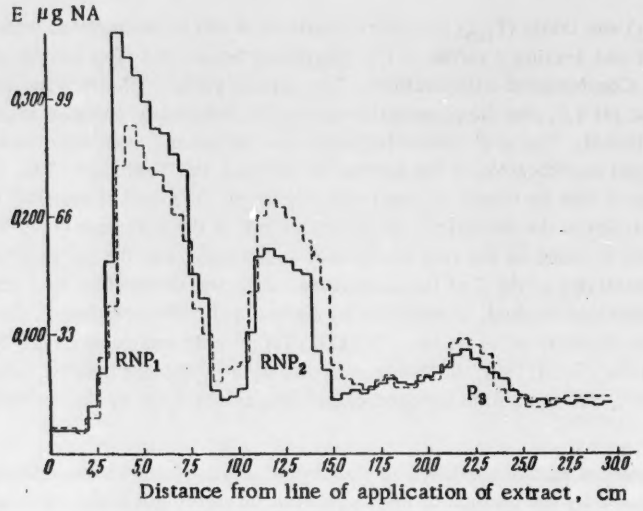

Figure. Electrophoretic separation of the ribonucleoproteins of Az. vinelandii. Continuous line – normal; broken line – chloramphenicol.

Since under the influence of chloramphenicol the dimensions of the Azotobacter cells increased considerably, all the analytical calculations were made not only per 10^9 cells, but also per mg dry weight of bacteria. The cells were counted by Vinogradskii's method.

EXPERIMENTAL RESULTS

The Action of Chloramphenicol on the Chemical Composition of the Cells of Azotobacter vinelandii

In Tables 1 and 2 we present figures showing the quantitative content of various substances in Az. vinelandii, and the RA of the P of the phosphorus compounds and of the C^{14}-protein. It is clear from these figures that under the action of chloramphenicol, the total RNA content when calculated per 10^9 cells was unchanged, and when calculated per 1 mg dry weight it was slightly decreased (Table 1). With equal exposures of the cells with radioactive orthophosphate the RA of the phosphorus of the total RNA in the inhibited and normal cells was equal (Table 2).

The DNA content of the culture inhibited by the antibiotic, when calculated per individual cell, changed only very slightly, but calculated per dry weight of bacteria, it altered quite considerably (Table 1). It was characteristic that under these circumstances the velocity of incorporation of P^{32} in the DNA was halved, which may be some indication of the considerable disturbance of the mechanism of its synthesis in the cell (Table 2). It must be pointed out that chloramphenicol inhibited particularly acutely the incorporation of C^{14}-glycine in the total protein of Azotobacter. It is clear from Table 2 that with equal exposure of the cells to C^{14}-glycine, the velocity of incorporation of C^{14} into the proteins of the inhibited culture was only one fifth that of the normal culture. If it is remembered that in normal conditions the number of cells of Azotobacter is roughly doubled in six hours, but in the presence of chloramphenicol it remains almost unchanged over the same period (although the cells increase in size), it will be obvious that the antibiotic essentially inhibits the synthesis of these substances.

TABLE 1. The Action of Chloramphenicol on the Qualitative Content of Certain Compounds of *Az. vinelandii*

Culture	N_{total}	NH_2 of free amino acids	P total	P total of free nucleotides	P of polyphosphates: soluble	P of polyphosphates: insoluble	P of phospholipids	Protein	RNA	DNA	Polysaccharide	Total lipids	O_2 utilized in μatoms per hr	Esterified P, in μatoms per hr	Dry weight of 10^9 cells, mg
	In μg/10^9 cells														
Normal	103.00	1.87	48.28	1.40	0.28	0.32	2.39	509.38	97.00	18.92	97.30	248.06	13.0	16.0	1.08
Inhibited	104.20	2.80	45.60	1.60	2.15	0.30	2.40	508.70	97.00	18.00	220.40	223.21	12.5	14.4	1.38
	In μg/mg dry weight										% of normal				
Normal	95.37	1.73	44.70	1.30	0.26	0.30	2.19	471.64	89.90	17.54	90.10	229.68	100	100	—
Inhibited	75.51	2.03	32.62	1.16	1.56	0.22	1.74	369.00	70.25	13.00	159.42	161.74	96	90	—

The disturbance of the synthesis of protein and nucleic acids under the action of the antibiotics led to some increase in the content of acid-soluble nucleotides, and especially to a considerable accumulation of free amino acids when calculated per 10^9 cells (Table 1). In the logarithmic phase of development, with a high velocity of synthesis of organic substances in the Azotobacter cell, hardly any polyphosphates were formed, since under these circumstances all the energy of oxidative phosphorylation was expended on current processes of vital activity and, primarily, on the synthesis of proteins and nucleic acids [11]. In the case of the action of chloramphenicol, while normal respiration and an almost normal level of oxidative phosphorylation were retained (Table 1), the processes of synthesis in the Azotobacter cell (for example, the synthesis of protein and DNA) was severely disturbed (Table 2). The disturbance of synthesis of a series of substances led to a reduction in the expenditure of energy normally going to form ATP, as a result of which there was a large (sevenfold) increase in the polyphosphate content. These polyphosphates, however, were accumulated not in the form of physiologically active, high-molecular compounds, but as acid-soluble polyphosphates of lower molecular weight. The presence of such a process in the Azotobacter cell was also demonstrated by the higher RA of the soluble polyphosphates in the inhibited culture than in the normal. The process of degradation of the primarily formed acid-insoluble polyphosphates was especially marked in the chloramphenicol-inhibited culture after incubation for 30 minutes in a radioactive medium; in these conditions the RA of the soluble and insoluble polyphosphates became roughly the same (Table 2). The RA of the acid-soluble phosphorylated carbohydrates was somewhat increased under the influence of the antibiotic, which could indicate the intensification of the carbohydrate metabolism of Azotobacter under these conditions (Table 2).

In fact, the content of intracellular polysaccharide in the culture inhibited with chloramphenicol was more than doubled (Table 1). The total quantity of lipids in the Azotobacter cell was unchanged by the action of chloramphenicol. However, on the basis of the appreciable fall in the RA of the P of the phospholipids in the inhibited culture, it can be assumed that chloramphenicol to some degree suppressed the synthesis of phospholipids.

Thus in the Azotobacter culture treated with chloramphenicol, the considerable inhibition of several substances and, in particular, of protein, DNA and phospholipids, was compensated by an increased formation of intracellular polysaccharides.

TABLE 2. The Action of Chloramphenicol on the Incorporation of Radioactive Phosphorus into Certain Phosphorus Compounds and of C^{14}-Glycine into the Protein of Az. vinelandii (in imp/min/μg P)

Time of incorporation of P^{32} or C^{14}, min	P of acid-soluble compounds						P of acid-insoluble compounds				Incorp of C^{14} in protein, imp/min	
	P_{total}	ortho-phosphate	soluble poly-phosphates	Nucleotides		phospho-rylated carbo-hydrates	insoluble poly-phosphates	P-RNA	P-DNA	P of phos-pholipids	per 1 mg protein	per 10^9 cells
				P_{lab} "ATP-ADP"	P_{total}							
Normal												
5	63.2	95.4	0	68.0	48.1	27.2	86.8	52.3	17.5	13.1	583.1	297.0
15	77.1	131.7	14.2	100.2	65.0	38.4	122.1	61.6	19.1	15.8	981.9	417.0
30	83.6	185.1	27.4	145.6	98.7	43.1	163.9	80.0	20.0	26.3	1598.0	814.0
Chloramphenicol												
5	44.1	90.6	48.1	65.4	45.3	34.5	87.4	52.7	-	8.6	164.3	83.6
15	50.3	127.3	83.7	96.2	61.8	42.3	122.3	65.4	9.9	13.3	164.0	83.2
30	61.9	150.5	113.4	137.3	95.1	56.7	118.0	81.6	12.0	18.5	299.0	152.1

The Action of Chloramphenicol on the Qualitative Composition of the Nucleic Acids of Az. vinelandii

There are reports in the literature that small concentrations of chloramphenicol cause marked stimulation of the synthesis of a qualitatively new RNA in certain bacteria [2,4,6-23]. It will be seen from Table 1 that in Az. vinelandii no excess accumulation of total RNA in the cell took place by the action of chloramphenicol in a concentration of $5 \cdot 10^{-4}$M. It might have been expected from these reports cited above, however, that some change could have occurred in the quality of the RNA in the inhibited Azotobacter culture. In order to test this hypothesis we carried out an investigation of the nucleotide composition of the total RNA and of the velocity of metabolism of the P of its component ribonucleotides. During the study of the nucleotide composition of the total RNA in the normal and chloramphenicol-inhibited cultures of Azotobacter, no appreciable difference was found (Table 3). Similar results were obtained by other workers [24,25]. The intensity of metabolism of the P of the mononucleotides of the total RNA after exposure for 5, 15 and 30 minutes was roughly the same in normal conditions and after treatment with chloramphenicol (Table 4).

It is an interesting fact that during brief incubation with radioactive orthophosphate (up to 30 minutes) the values of the RA of all four mononucleotides of the total RNA were very different. The highest radioactivity was shown by adenylic acid, average values by guanylic and cytidylic, and the least by uridylic acid (Table 4). In our previous work [11] we showed that during prolonged incubation with radioactive orthophosphate (48 hours) an equalization of the intensity of metabolism of the P-ribonucleotides of the total RNA of Azotobacter takes place, and that, moreover, the relative activity of their phosphorus corresponds to their molar percentage.

Chloramphenicol strongly suppressed the synthesis of DNA of Azotobacter (Table 2), although the nucleotide composition of the total DNA in the inhibited culture was the same as in the normal (Table 5).

Electrophoretic Separation of Ribonucleoproteins of Az. vinelandii

The absence of quantitative changes, and also the resemblance between the nucleotide composition of the total RNA in normal and chloramphenicol-inhibited cells of Az. vinelandii do not exclude the possibility that they are not homogeneous. In order to test this hypothesis, we attempted to separate the

TABLE 3. Nucleotide Composition of the Total RNA of Normal and Chloramphenicol-Inhibited Cultures of Az. vinelandii

Culture	Molar percentages				Pu/Py	G+U/A+C	G+C/A+U
	G	A	C	U			
Normal	30.18	24.92	25.48	19.42	1.22	0.99	1.26
Inhibited	30.61	24.00	25.40	19.99	1.20	1.04	1.27

Note. Here and in Table 4: G = guanylic, A = adenylic, C = cytidylic and U = uridylic acids; Pu = purine nucleotides; Py = pyrimidine nucleotides.

TABLE 4. Relative Activity of P^{32} of Nucleotides of Total RNA in Normal and Chloramphenicol-Inhibited Cultures of Az. vinelandii (In imp/min/μg P)

Time of incorporation, min	G	A	C	U	P-RNA	Time of incorporation	G	A	C	U	P-RNA
	Normal						Chloramphenicol				
5	27.4	134.8	32.7	17.2	52.3	5	29.0	131.1	33.0	15.0	52.7
15	43.5	143.6	37.4	21.7	61.6	15	44.1	147.2	38.3	30.0	65.4
30	47.9	196.3	41.8	35.7	80.8	30	48.7	198.0	44.1	33.0	81.6

ribonucleoproteins of normal, inhibited and restored cultures of Azotobacter, incubated with radioactive orthophosphate for two hours, into their individual components, so that by measurement of the velocity of their phosphorus metabolism some idea might be obtained of their qualitative differences.

TABLE 5. Nucleotide Composition of the DNA of Normal and Chloramphenicol-Inhibited Cultures of Az. vinelandii

Culture	Molar percentages				Pu/Py	G+T/A+C	G+C/A+T
	G	A	C	T			
Normal	27.37	22.08	28.88	21.67	0.98	0.97	1.28
Inhibited	27.56	22.42	28.20	21.82	1.00	0.98	1.26

Note. G = guanine; A = adenine; C = cytosine; T = thymine; Pu = purine bases; Py = pyrimidine bases.

By means of the method of electrophoresis in a starch gel, the nucleoporteins of Az. vinelandii could be separated into two large ribonucleoprotein peaks (RNP_1 and RNP_2) and one small peak (P_3), consisting mainly of degraded DNA, free from protein (figure). The electrophoretic mobility of both RNP_1 and RNP_2 in the normal and inhibited cells was identical, which indicated that the corresponding ribonucleotide complexes in these cultures are to some extent equivalent. The action of chloramphenicol led principally to a change in the quantitative proportions of RNP_1 and RNP_2, namely to a rise in the RNP_2 peak and a fall in the RNP_1 peak (Table 6).

Comparison of the values of the RA of P-RNP_1 and P-RNP_2 in the normal culture showed a certain difference between them. The 50% increase in the value of the RA of P-RNP_2 over that of the RA of P-RNP_1 in the inhibited

culture could be the result of a selective intensification of the synthesis of RNP_2, and conversely the appreciable fall in the RA of P-RNP_1 in the inhibited culture by comparison with the normal could indicate the suppression of its synthesis.

It is interesting that the RA of P-RNP_2 increased perceptibly under the action of chloramphenicol, in spite of the depressing effect of the antibiotic. This is due to the fact that in the presence of chloramphenicol an intensification of the synthesis of RNP_2 was observed, as a result of which a preferential incorporation or radioactive orthophosphate into this particular ribonucleoprotein occurred.

The intensity of metabolism of phosphorus thus differed to some extent during the two hours in the two ribonucleoproteins of the normal culture, which evidently could be a sign of their metabolic nonhomogeneity. The difference between the values of the RA of the phosphorus of these same complexes in the inhibited culture could indicate a difference in the velocity of their synthesis in the cell under the influence of the antibiotic.

The RA of the phosphorus of the DNA of the third peak (P_3) fell by one third (Table 6). This was in good agreement with our previous findings relating to the metabolism of P-DNA in inhibited and normal whole cells of Az. vinelandii (Table 2).

Since the nucleotide composition of the total RNA before and after treatment of the Azotobacter cells with chloramphenicol remained unchanged (Table 3), although the quantitative proportions of RNA_1 and RNA_2 in the cell in these two cases altered appreciably (Table 6), it may be considered that the nucleotide composition of the RNA_1 and RNA_2 was roughly identical.

It is possible that a more detailed study of the physical and chemical properties of these nucleic acids would reveal finer differences in their chemical structure.

TABLE 6. Electrophoretic Study of the Nucleic Acids of Az. vinelandii 2 ml of Extract Tested

Electrophoretic compound	Quantity of nucleic acid in nucleoprotein		Quantity of protein in nucleoprotein, μg	RNA / protein	Relative activity of nucleic acid P, imp/min/μg P
	μg	in % of total RNA			
			Normal		
RNP_1	1169	77	1200	1.0	248
RNP_2	349	23	268	1.3	200
Mixed P_3	227	–	–	–	108
			Chloramphenicol		
RNP_1	977	48	980	1.0	183
RNP_2	689	42	401	1.7	300
Mixed P_3	249	–	–	–	71
			Restored culture		
RNP_1	1002	75	930	1.1	–
RNP_2	325	25	250	1.3	–
Mixed P_3	233	–	–	–	–

SUMMARY

Chloramphenicol ($5 \cdot 10^{-4}$M) greatly inhibits the incorporation of C^{14}-glycine into proteins and of orthophosphate into DNA, thereby demonstrating derangement of synthesis of these compounds in the cells of Azotobacter vinelandii. Inhibition of protein and DNA synthesis by chloramphenicol results in an increase of low-molecular compounds in the cell, viz. of acid-soluble amino acids, mononucleotides and of soluble polyphosphates. The amount of intracellular polysaccharide is increased by chloramphenicol almost twofold. In the Azotobacter cells inhibited by the antibiotic, the level of RNA and the rate of phosphorus exchange do not change as compared with the normal. The nucleotide composition of DNA as well as that of total RNA and the specific activity of P of its component ribonucleotides are likewise similar in the normal and inhibited culture.

By starch-gel electrophoresis the ribonucleoproteins of Az. vinelandii have been separated into two major components somewhat differing in the rate of phosphorus exchange. Chloramphenicol calls forth a change in the ratio of the two ribonucleoproteins by increasing mostly the content of the component with a higher electrophoretic mobility.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Émbriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

* BRITISH-AMERICAN TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Ekspti. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

Soviet Journals Available in Cover-to-Cover Translation

ABBREVIATION	RUSSIAN TITLE	TITLE OF TRANSLATION	PUBLISHER	TRANSLATION BEGAN Vol.	Issue	Year
AÉ	Atomnaya énergiya	Soviet Journal of Atomic Energy	Consultants Bureau	1	1	1956
Akust. zh.	Akusticheskii zhurnal	Soviet Physics – Acoustics	American Institute of Physics	1	1	1955
	Antibiotiki	Antibiotics	Consultants Bureau	4	1	1959
Astr(on). zh(urn).	Astronomicheskii zhurnal	Soviet Astronomy–AJ	American Institute of Physics	34	1	1957
Avto(mat). svarka	Avtomaticheskaya svarka	Automatic Welding	British Welding Research Association (London)		1	1959
	Avtomatika i Telemekhanika	Automation and Remote Control	Instrument Society of America	27	1	1956
	Biofizika	Biophysics	National Institutes of Health*		1	1957
	Biokhimiya	Biochemistry	Consultants Bureau	21	1	1956
Byull. éksp(erim). biol. i med.	Byulleten' éksperimental'noi biologii i meditsiny	Bulletin of Experimental Biology and Medicine	Consultants Bureau	41	1	1959
DAN (SSSR) / Dokl(ady) AN SSSR	Doklady Akademii Nauk SSSR	The translation of this journal is published in sections, as follows:				
		Life Sciences: Doklady Biochemistry Section	American Institute of Biological Sciences	106	1	1956
		Life Sciences: Doklady Biological Sciences Sections (Includes: Anatomy, biophysics, cytology, ecology, embryology, endocrinology, evolutionary morphology, genetics, histology, hydrobiology microbiology, morphology, parasitology, physiology, zoology sections)	American Institute of Biological Sciences	112	1	1957
		Life Sciences: Doklady Botanical Sciences Sections (Includes: Botany, phytopathology, plant anatomy, plant ecology, plant embryology, plant physiology, plant morphology sections)	American Institute of Biological Sciences	112	1	1957
		Chemical Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Chemical Technology	Consultants Bureau	106	1	1956
		Chemical Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Chemistry	Consultants Bureau	106	1	1956
		Chemical Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Physical Chemistry	Consultants Bureau	112	1	1957
		Earth Sciences: Doklady Earth Sciences Sections (Includes: Geochemistry, geology, geophysics, hydrogeology, mineralogy, paleontology, petrography, permafrost sections)	American Geological Institute	124	1	1959
		Earth Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Geochemistry	Consultants Bureau	106-123	1-6	1957-1958
		Earth Sciences: Proceedings of the Academy of Sciences of the USSR, Sections: Geology	Consultants Bureau	106-123	1-6	1957-1958
		Mathematics: Doklady Soviet Mathematics	The American Mathematics Society	131	1	1961
		Physics: Soviet Physics–Doklady (Includes: Aerodynamics, astronomy, crystallography, cybernetics and control theory, electrical engineering, energetics, fluid mechanics, heat engineering, hydraulics, mathematical physics, mechanics, physics, technical physics, theory of elasticity sections)	American Institute of Physics	106	1	1956
		Physics: Proceedings of the Academy of Sciences of the USSR, Applied Physics Sections (does not include mathematical physics or physics sections)	Consultants Bureau	106-117	1	1956-1957
Derevoobrabat. prom-st'.	Derevoobrabatyvayushchaya promyshlennost'	Wood Processing Industry	Timber Development Association (London)		9	1959
	Élecktrosvyaz	Telecommunications	Massachusetts Institute of Technology*		1	1957
Éntom(ol). oboz(renie)	Éntomologicheskoe obozrenie	Entomological Review	American Institute of Biological Sciences	38	1	1959
Farmakol. (i) toksikol(ogiya)	Farmakologiya i toksikologiya	Pharmacology and Toxicology	Consultants Bureau	20	1	1957
FMM	Fizika metallov i metallovedenie	Physics of Metals and Metallography	Acta Metallurgica*	5	1	1957
Fiziol. zhurn. SSSR (im. Sechenova)	Fiziologicheskii zhurnal im. I. M. Sechenova	Sechenov Physiological Journal USSR	National Institutes of Health*		1	1957
Fiziol(ogiya) rast.	Fiziologiya rastenii	Plant Physiology	American Institute of Biological Sciences	4	1	1957
	Geokhimiya	Geochemistry	The Geochemical Society		1	1958
FTT	Fizika tverdogo tela	Soviet Physics–Solid State	American Institute of Physics	1	1	1959
Izmerit. tekh(nika)	Izmeritel'naya tekhnika	Measurement Techniques	Instrument Society of America		1	1959
Izv. AN SSSR, O(td). Kh(im). N(auk)	Izvestiya Akademii Nauk SSSR: Otdelenie khimicheskikh nauk	Bulletin of the Academy of Sciences of the USSR: Division of Chemical Sciences	Consultants Bureau		1	1952

continued

Izv. AN SSSR, O(td). T(ekhn). N(auk): Met(all). i top.	(see Met. i top.)				
Izv. AN SSSR Ser. fiz(ich).	Izvestiya Akademii Nauk SSSR: Seriya fizicheskaya	Bulletin of the Academy of Sciences of the USSR: Physical Series	Columbia Technical Translations		1 1954
Izv. AN SSSR Ser. geofiz.	Izvestiya Akademii Nauk SSSR: Seriya geofizicheskaya	Bulletin (Izvestiya) of the Academy of Sciences USSR: Geophysics Series	American Geophysical Union		1 1954
Izv. AN SSSR Ser. geol.	Izvestiya Akademii Nauk SSSR: Seriya geologicheskaya	Izvestiya of the Academy of Sciences of the USSR: Geologic Series	American Geological Institute		1 1958
Kauch. i rez.	Kauchuk i rezina	Soviet Rubber Technology	Research Association of British Rubber Manufacturers	18	1 1959
	Kinetika i kataliz	Kinetics and Catalysis	Consultants Bureau	1	3 1960
	Koks i khimiya	Coke and Chemistry USSR	Coal Tar Research Association (Leeds, England)		1 1958
Kolloidn. zh(urn).	Kolloidnyi zhurnal	Colloid Journal	Consultants Bureau	14	1 1952
	Kristallografiya	Soviet Physics – Crystallography	American Institute of Physics	2	1 1957
Metalov. i term. obrabot. metal.	Metallovedenie i termicheskaya obrabotka metallov	Metal Science and Heat Treatment of Metals	Acta Metallurgica	6	1 1958
	Metallurg	Metallurgist	Acta Metallurgica		1 1957
Met. i top.	Metallurgiya i topliva	Russian Metallurgy and Fuels	Eagle Technical Publications		1 1960
Mikrobiol.	Mikrobiologiya	Microbiology	American Institute of Biological Sciences	26	1 1957
OS	Optika i spektroskopiya	Optics and Spectroscopy	American Institute of Physics		6 1959
	Pochvovedenie	Soviet Soil Science	American Institute of Biological Sciences		1 1958
	Priborostroenie	Instrument Construction	British Scientific Instrument Research Association		1 1959
Pribory i tekhn. éks(perimenta)	Pribory i tekhnika éksperimenta	Instruments and Experimental Techniques	Instrument Society of America		1 1957
Prikl. matem. i mekh.	Prikladnaya matematika i mekhanika	Applied Mathematics and Mechanics	American Society of Mechanical Engineers		1 1958
PTÉ	(see Pribory i tekhn. éks.)				
	Problemy Severa	Problems of the North	National Research Council of Canada		
Radiotekh.	Radiotekhnika	Radio Engineering	Massachusetts Institute of Technology*	12	1 1957
Radiotekh. i élektronika	Radiotekhnika i élektronika	Radio Engineering and Electronics	Massachusetts Institute of Technology*	2	1 1957
	Stanki i instrument	Machines and Tooling	Production Engineering Research Assoc.		1 1959
	Stal'	Stal (In English)	Iron and Steel Institute		1 1959
Stek. i keram.	Steklo i keramika	Glass and Ceramics	Consultants Bureau	13	1 1956
Svaroch. proiz-vo	Svarochnoe proizvodstvo	Welding Production	British Welding Research Association		4 1959
Teor. veroyat. i prim.	Teoriya veroyatnostei i ee primenenie	Theory of Probability and Its Applications	Society for Industrial and Applied Mathematics		1 1956
Tsvet. Metally	Tsvetnye metally	Nonferrous Metals	Primary Sources		1 1960
UFN	Uspekhi fizicheskikh Nauk	Soviet Physics – Uspekhi (partial translation)	American Institute of Physics	66	1 1958
UKh	Uspekhi khimii	Russian Chemical Reviews	The Chemical Society (London)		1 1960
UMN	Uspekhi matematicheskikh nauk	Russian Mathematical Surveys	London Mathematical Society	15	1 1960
Usp. fiz. nauk	(see UFN)				
Usp. khim(ii)	(see UKh)				
Usp. matem. nauk	(see UMN)				
Usp. sovr. biol.	Uspekhi sovremennoi biologii	Russian Review of Biology	Oliver and Boyd		48 1959
Vest. mashinostroeniya	Vestnik mashinostroeniya	Russian Engineering Journal	Production Engineering Research Assoc.		4 1959
Vop. gem. i per. krovi	Voprosy gematologii i perelivaniya krovi	Problems of Hematology and Blood Transfusion	National Institutes of Health*		1 1957
Vop. onk.	Voprosy onkologii	Problems of Oncology	National Institutes of Health*		1 1957
Vop. virusol.	Voprosy virusologii	Problems of Virology	National Institutes of Health*		1 1957
Zav(odsk). lab(oratoriya)	Zavodskaya laboratoriya	Industrial Laboratory	Instrument Society of America	25	1 1959
ZhAKh Zh. anal(it). khimii	Zhurnal analiticheskoi khimii	Journal of Analytical Chemistry USSR	Consultants Bureau	7	1 1952
ZhETF Zh. éksperim. i teor. fiz.	Zhurnal éksperimental'noi i teoreticheskoi fiziki	Soviet Physics–JETP	American Institute of Physics	28	1 1955
ZhFKh Zh. fiz. khimii	Zhurnal fizicheskoi khimii	Russian Journal of Physical Chemistry	The Chemical Society (London)		7 1959
ZhMÉI Zh(urn). mikrobiol. épidemiol. i immunobiol.	Zhurnal mikrobiologii, épidemiologii i immunobiologii	Journal of Microbiology, Epidemiology and Immunobiology	National Institutes of Health*		1 1957
ZhNKh Zh(urn). neorgan(ich). khim(ii)	Zhurnal neorganicheskoi khimii	The Russian Journal of Inorganic Chemistry	The Chemical Society (London)		1 1959
ZhOKh Zh(urn). obshch(ei) khimii	Zhurnal obshchei khimii	Journal of General Chemistry USSR	Consultants Bureau	19	1 1949
ZhPKh Zh(urn). prikl. khimii	Zhurnal prikladnoi khimii	Journal of Applied Chemistry USSR	Consultants Bureau	23	1 1950
ZhSKh Zh(urn). strukt. khimii	Zhurnal strukturnoi khimii	Journal of Structural Chemistry	Consultants Bureau	1	1 1960
ZhTF Zh(urn). tekhn. fiz.	Zhurnal teknicheskoi fiziki	Soviet Physics–Technical Physics	American Institute of Physics	26	1 1956
Zh(urn). vyssh. nervn. deyat. (im. Pavlova)	Zhurnal vysshei nervnoi deyatel'nosti (im. I. P. Pavlova)	Pavlov Journal of Higher Nervous Activity	National Institutes of Health*		1 1958

*Sponsoring organization. Translation through 1960 issues is a publication of Pergamon Press.

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2,2'-Methylenedifuran
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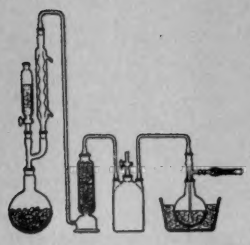

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